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HIV-1 and Cocaine: Motivation and Synaptic Function

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HIV-1 AND COCAINE: MOTIVATION AND SYNAPTIC FUNCTION

by

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DEDICATION

I dedicate this to my entire family (Bertrands, Baileys, Robinsons, Stanulonises), without whom I do not think I could have made it through my education. But I especially dedicate this to my parents, who have always allowed and encouraged me to follow my dreams. Without you, none of this would have been possible.

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ABSTRACT

Molecular and behavioral assays were used to examine the effects of HIV-1 on synaptodendritic integrity, motivated behavior and the therapeutic potential of natural isoflavones in ameliorating both synaptodendritic injury *in vitro* and changes in motivation *in vivo.* The overarching hypothesis of the present dissertation is twofold: 1. HIV-1 causes a decrease in motivation for sucrose and cocaine and the change in motivational state is driven by compromised synaptic integrity as evaluated by F-actin and DAT function and, 2. Treatment with phytoestrogens will prevent synaptodendritic damage, normalize the internal motivational state of the HIV-1 Tg rat for sucrose and cocaine, and alter dendritic spine morphology.

Primary neuronal cell cultures were utilized to evaluate the effects of HIV-1 Tat and cocaine on synaptodendritic integrity and the therapeutic potential of phytoestrogens. We demonstrate that the cysteine rich domain of Tat is required to initiate synaptodendritic injury, and damage can be prevented by pre-treatment with phytoestrogens daidzein, liquiritigenin, and equol in an estrogen receptor dependent manner. Addition of daidzein and liquiritigenin to the cell culture medium following Tat exposure resulted in a significant restoration of the fine network which was an estrogen receptor mediated mechanism. Simultaneous treatment with Tat and cocaine, at independently non-damaging concentrations, resulted in significant synaptodendritic

injury. Tat + cocaine induced damage is prevented by S/R-equol treatment in an estrogen receptor beta dependent mechanism.

The HIV-1 transgenic rat was utilized to evaluate the effects of HIV-1 on motivated behavior for sucrose and cocaine, and the ability of equol treatment to potentially modulate changes in goal-directed behavior. HIV-1 Tg rats respond less overall for sucrose (0-30%; w/v) and may be more sensitive to cocaine. Equol treatment resulted in a differential response to sucrose and cocaine in both HIV-1 Tg and F344 rats. Control animals respond in a functionally linear manner for higher sucrose concentrations, but equol treatment produced a quadratic response. Equol increased cocaine intake in F344 rats but resulted in a decrease in responding in HIV-1 Tg rats. Finally, equol treatment shifted choice behavior in F344 animals. Together, these studies indicate equol may be a beneficial treatment for HIV-1+ individuals, but may not be suitable for seronegative individuals.

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ART..... Anti-retroviral therapy

cART.....Combined anti-retroviral therapy

DA.....Dopamine

DAT ..... Dopamine Transporter

ER (α,β).....Estrogen Receptor

F-actin .....Filamentous Actin

FR.....Fixed Ratio

F344-E.....F344 Equol treated rat

F344-S.....F344 Sucrose treated rat

G-actin.....Globular actin

GPR 30.....G-Protein coupled Receptor 30

HAND ..... HIV-1 Associated Neurocognitive Disorders

HAD..... HIV-1 Associated Dementia

HIV-1+ ..... HIV-1 positive

HIV-1 Tg..... HIV-1 Transgenic rat

HIV-1-E .....HIV-1 Tg equol treated rat

HIV-1-S.....HIV-1 Tg sucrose treated rat

MCMD.....Minor cognitive-motor disorder

NAc .....Nucleus Accumbens

OVX..... Ovariectomized

PR..... Progressive Ratio

RE ..... R-Equol

SE ..... S-Equol

CHAPTER 1

INTRODUCTION

HIV-1: Dopamine, apathy, and motivation

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that currently infects 34 million people world-wide, with roughly 1.5 million living in the United States (World Health Organization, 2011). HIV-1 enters the CNS shortly after infection (Resnick, Berger, Shapshak, & Tourtellotte, 1988; Nath, 2002), where it can be found in microglia and a small percentage of astrocytes (An, Groves, Gray, & Scaravilli, 1999; Ellis, Langford, & Masliah, 2007; Gonzalez-Scarano & Martin-Garcia, 2005). Once HIV-1 has infected the CNS, it remains unaffected by combined anti-retroviral therapy (cART) for the lifetime of the infected individual. The inability of cART to cross the BBB and the longevity of astrocytes make the brain important viral reservoir despite effective viral suppression in the periphery.

HIV-1 infection frequently results in a spectrum of neurocognitive deficits (HIV-1 associated neurocognitive disorders; HAND) that can range from mild (minor cognitive-motor disorder; MCMD) to severe (HIV-1 associated dementia; HAD) (Antinori et al., 2007). Following the implementation of cART there was a significant reduction in the incidence of HAD; however, the overall prevalence of HAND has not declined with more than 40% of HIV-1+ individuals exhibiting symptoms (Lindl, Marks, Kolson, & Jordan-Sciutto, 2010; Letendre, 2011). Despite the continued prevalence of HAND, there

are no therapeutic options for these individuals nor are the underlying mechanisms fully understood.

Depression frequently occurs in HIV-1+ individuals (Vance et al., 2014), however the severity of depressive symptoms are generally not found to be predictive of neurocognitive impairment (Goggin et al., 1997; Cysique et al., 2007). Depression and apathy are frequently comorbid disorders, however they can occur independently of one another (Marin, 1991; Marin, Firinciogullari, & Biedrzycki, 1993). Apathy is defined as a reduction in self-initiated, goal-directed behavior, or a lack of motivation. HIV-1 positive individuals consistently report higher incidence of apathy than seronegative controls (Kamat, Woods, Marcotte, Ellis, & Grant, 2012; Castellon, Hinkin, Wood, & Yarema, 1998a; Paul et al., 2005a). Elevated apathy scores are predictive of decreased medication adherence (Panos et al., 2014), increased cognitive complaints, diminished neurocognitive performance (Shapiro, Mahoney, Zingman, Pogge, & Verghese, 2013; Kamat et al., 2012), and suboptimal everyday functioning (Kamat et al., 2012; Kamat et al., 2013). Elevated levels of apathy are related alterations in learning efficiency and cognitive flexibility (Paul et al., 2005a), in addition to deficits in working memory performance (Castellon et al., 1998a) and executive function in HIV-1+ individuals (Castellon, Hinkin, & Myers, 2000).

Motivation is not considered a facet of cognition, however it is an important supporting factor for normal cognitive function (Keeler & Robbins, 2011). Due to the importance that motivation may play in supporting optimum cognitive functioning, and the correlation between motivation disturbance (Castellon et al., 2006; Castellon, Hinkin, Wood, & Yarema, 1998b), with cognitive function (Panos et al., 2014) in HIV-1+

individuals, evaluating the internal motivational state may be an important and predictive state to assess during HIV-1 infection to identify individuals at risk for cognitive impairment.

Dysfunction of, and damage, to the frontal-subcortical system is believed to play a role in manifestation of apathy (Masterman & Cummings, 1997; Tekin & Cummings, 2002). The mesocorticolimbic system encompasses several dopamine rich regions, which are known to be more susceptible to HIV-1 infection (Berger & Nath, 1997; Nath et al., 2000). HIV-1+ individuals display a sensitivity to dopamine receptor agonists (Berger & Nath, 1997), less dopamine in cerebral spinal fluid (Berger, Kumar, Kumar, Fernandez, & Levin, 1994), reduced dopamine transporter (DAT) binding in the striatum (Chang et al., 2008), lower TH levels in the susbstantia nigra (Silvers et al., 2006), and Parkinsonian-like symptoms (Tse et al., 2004). Apathy in HIV-1 positive individuals is associated with decreased performance on tasks known to involve the frontal-subcortical circuitry (Cole et al., 2007), and lower overall volume of the nucleus accumbens (Paul et al., 2005b). The hallmark features of HAND, e.g. poor memory and poor performance on executive function tasks, have been associated with decreased levels of dopamine and its metabolite, homovanillic acid, in individuals infected with HIV-1 (Di Rocco et al., 2000). In sum, the susceptibility of the dopaminergic system may play a role in the expression of apathy and executive function in HAND.

Neural systems that mediate the motivational features of goal-directed behavior are damaged by HIV-1 (Everitt & Robbins, 2013; Purohit, Rapaka, & Shurtleff, 2011). The striatum (caudate and putamen) of the basal ganglia receive major dopamine input from the ventral tegmental area and the substantia nigra, the mesocorticolimbic and

nigrostriatal dopamine systems, respectively. HIV-1 positive individuals exhibit a high viral load in the caudate (Kumar, Borodowsky, Fernandez, Gonzalez, & Kumar, 2007; Kumar et al., 2009a) and extensive atrophy in the caudate and putamen are reported in people exhibiting cognitive impairment and apathy (Paul et al., 2005a; Paul, Cohen, Navia, & Tashima, 2002; Hestad et al., 1993; Dal Pan et al., 1992; Ances et al., 2006). The mesolimbic portion of the dopamine system is associated with motivation, and the nigrostriatal system with the organization of motor systems to complete, and consummate responding, together resulting in goal-directed behavior. Mesolimbic dopamine, which is associated with reward, mediates several aspects of motivation, specifically related to activity, such as behavioral activation (Koob, Riley, Smith, & Robbins, 1978), approach behavior (Robinson & Berridge, 2003; Nicola, 2010) and exertion of effort (Salamone, Cousins, & Bucher, 1994) particularly regarding food-maintained operant responding (Salamone & Correa, 2012). The mesolimbic dopamine system is also responsible for partially mediating drug-maintained responding, and highly reinforcing stimuli such as cocaine have been investigated extensively (Roberts, Morgan, & Liu, 2007; Espana & Jones, 2013; Nader, Balster, & Henningfield, 2014). Thus, goal-directed behavior relies on mesolimbic and nigrostriatal dopamine for normal function, and HIV-1 produces extensive neuropathology throughout the striatum, the major dopamine output regions of these systems.

HIV-1: Viral proteins and their effects on the CNS

HIV-1 infection has been primarily studied as an immunological process since its emergence in the 1980s. The virus is made up of 9 distinct genes which code for proteins required for successful entry into the cell, and replication within the cell. There are three

structural genes core (*gag*), polymerase (*pol*), and envelope (*env*); and six regulatory genes the transactivator of transcription (*tat*), regulator of viral RNA splicing and transport (*rev*), viral protein U (*vpu*), viral protein R (*vpr*), negative factor (*nef*), and viral infectivity protein (*vif*) (Nath, 2002; Pavlakis & Felber, 1990). Each of these genes plays an important role in infectivity, replication, and maintenance of the virus.

Although HIV-1 enters the brain early in infection and induces neuronal death and damage (Everall et al., 1999; Masliah et al., 1997), it does not directly infect neurons. Each HIV-1 gene codes for a specific protein(s) that generally correspond in name, and of these proteins Tat, glycoprotein 120 and 41(gp120, gp41; encoded by *env*), Rev, Vpr, and Nef are known to be neurotoxic (Nath, 2002; Aksenova, Aksenov, Adams, Mactutus, & Booze, 2009; Agrawal et al., 2010; Bansal et al., 2000). The neurotoxic activities of these proteins play a major role in the neuronal damage and death that result in HAND.

HIV-1 Tat is one of the most well studied neurotoxic proteins produced by HIV-1. Tat is a non-structural protein that exists from 86-101 amino acids that is encoded by two axons (Nath, 2002). The viral function of Tat is to stabilize the transcription process and more efficiently replicate the virus (Dayton, Sodroski, Rosen, Goh, & Haseltine, 1986; Debaisieux, Rayne, Yezid, & Beaumelle, 2012; Gatignol & Jeang, 2000; Pugliese, Vidotto, Beltramo, Petrini, & Torre, 2005). Unlike the majority of HIV-1 proteins, Tat is released into the extracellular space by infected glial cells (Tardieu, Hery, Peudenier, Boespflug, & Montagnier, 1992; Chang, Samaniego, Nair, Buonaguro, & Ensoli, 1997; Ensoli et al., 1993) where it interacts with neurons to produce oxidative stress (Aksenov et al., 2001; Kruman, Nath, & Mattson, 1998), and calcium channel imbalance (Kruman et al., 1998). HIV-1 Tat also modulates NMDA receptors (Li et al., 2008), D1 receptors

(Aksenov, Aksenova, Mactutus, & Booze, 2012; Silvers, Aksenova, Aksenov, Mactutus, & Booze, 2007), and the dopamine transporter (DAT) (Midde, Gomez, & Zhu, 2012; Midde et al., 2013). Together the extracellular actions of HIV-1 Tat result in neuronal cell death (Adams, Aksenova, Aksenov, Mactutus, & Booze, 2012; Aksenov et al., 2012; Bonavia et al., 2001; Kruman et al., 1998; Mattson, Haughey, & Nath, 2005) and dendritic injury (Bertrand, Aksenova, Mactutus, & Booze, 2013; Kim, Martemyanov, & Thayer, 2008a; Shin, Kim, & Thayer, 2012a) in dopamine rich areas.

DAT, which regulates dopaminergic tone via reuptake, is also compromised by HIV-1. Patients exhibiting HAND show reductions in DAT throughout the putamen and nucleus accumbens (Chang et al., 2008; Wang et al., 2004). Furthermore, animal models indicate that HIV-1 viral proteins, such as Tat and gp120, are neurotoxic and impair DAT function (Aksenov, Aksenova, Silvers, Mactutus, & Booze, 2008; Ferris, Frederick-Duus, Fadel, Mactutus, & Booze, 2009a; Zhu, Mactutus, Wallace, & Booze, 2009; Zhu, Ananthan, Mactutus, & Booze, 2011). Tat mediates these effects by allosterically modulating the DAT through protein-protein interactions (Zhu et al., 2011). Although HIV-1 protein/DA interactions are not the sole mediator of death and damage in the CNS, it is clear that HIV-1 viral proteins produce damage to the mesolimbic and nigrostriatal DA systems, and that such interactions play an important role in the development of HAND.

HIV-1 and Cocaine Use

Cocaine is a highly addictive stimulant drug extracted from the leaves of the coca plant, *Erythroxylon coca*. *Erythroxylon coca* use originated in South America, where Andean tribes have chewed the leaves for increased alertness, anti-appetitive properties,

and a general sense of wellbeing for thousands of years (Schwartz, Rezkalla, & Kloner, 2010; Maraj, Figueredo, & Lynn, 2010). Coca leaves are also rich in vitamins and minerals, and do not produce the stimulant effects of cocaine (Stolberg, 2011). Although there were several attempts at exporting the coca leaf, it rotted quickly and lost any medicinal value during exportation. In 1858, German chemist Albert Niemann managed to separate cocaine from the coca leaf (Stolberg, 2011). The initial extract from *Erythroxylon coca* is alkaloid, and is dissolved in hydrochloric acid to form the water-soluble salt cocaine hydrochloride and could easily be shipped over long distances (Das, 1993).

In the late 19th and early 20th century cocaine was thought to be a remedy for many medical maladies, such as fatigue, toothaches, headaches, hay fever, seasickness, and even vomiting during pregnancy. Cocaine was first used as a local topical anesthetic in 1884 (Maraj et al., 2010), and William Halsted furthered this practice when finding that injecting cocaine under the skin was more effective and lessened bleeding (Stolberg, 2011). Unfortunately, it was believed that the powder form of cocaine was as non-addictive as the leaf form of the drug. During this time, pure cocaine was sold in the open market and popular beverages, such as the wine Vin Mariani and Coca-Cola, were laced with cocaine for 'health benefits' (Das, 1993).

However, signs of abuse soon began to show in the general public. Death, deterioration of the nasal cavity, and signs of dependence began to surface in users nationwide. In 1914 the U.S. government banned use of cocaine except when used by a physician as a local anesthetic as public knowledge of the toxic side effects of cocaine and the abuse potential of cocaine spread (Das, 1993). Today, under the U.S. Controlled

Substance Act of 1970 cocaine is a schedule II drug, meaning that cocaine has a high potential for abuse and its use may lead to severe physical and psychological dependence; however, cocaine still has accepted medical use as a local anesthetic.

Despite federal regulation, cocaine abuse continues to be a national health crisis: in 2009 and 2010 4.7 million Americans age 12 and older indicated that they had abused cocaine in the last month (SAMHSA, 2012). Cocaine use is responsible for 24% of drug-related hospital visits every year, presenting a significant financial burden on users and the welfare system of users who cannot afford health care coverage (NIDA, 2010). Additionally, risky behaviors and intravenous injection of cocaine put users at a higher risk for contracting HIV/AIDS, and hepatitis C, highlighting the need to treat current addicts.

Despite the prevalence of severe risks associated with HIV-1+ cocaine use, there are currently no effective pharmacotherapies, and although behavioral therapy is effective for drug abuse, pharmacotherapeutic options may enhance outcomes (NIDA, 2010). Therapeutic intervention in addiction may be an important step in not only increasing the quality of life of these individuals, it may prevent further HIV-1 transmission. Large pharmaceutical companies do not have the financial incentive to develop therapeutics for cocaine addiction or HIV-1+ cocaine addicts, because most addicts are of low socioeconomic status and not likely to have the ability to afford a new drug (NIDA, 2010). However, natural plant derived therapies have been used for thousands of years by a variety of cultures for various ailments. Several naturally occurring compounds have anti-viral and anti-inflammatory properties (Harborne & Williams, 2000; Vrijsen, Everaert, & Boeye, 1988). Within the last 20 years, various natural therapies have been

tested in clinical trials for drug addiction with mixed efficacy, mostly due to the mixtures used in these trials (Lu et al., 2009). Presently, investigations into natural compounds have resulted in the identification of multiple active constituents. The identification of active components coupled with the increase in the knowledge of neurobiological mechanisms of drug addiction and HIV-1 neurocognitive disorders allows researchers to better identify useful plant derived compounds for treatment of drug abuse and HAND.

More than 30% of HIV-1+ individuals in the US admit to using illicit drugs regularly (Korthuis et al., 2008), with up to half displaying neurocognitive impairment (Devlin et al., 2012), and cocaine is one of the most commonly abused drugs among HIV-1+ individuals (Korthuis et al., 2008). Although the increase in prevalence and severity of neurocognitive deficits in HIV-1+ drug users is well known (Nath, 2010; Purohit et al., 2011), preclinical studies have yielded complex answers in regards to an underlying mechanism. Administration of dopaminergic drugs, like L-DOPA or selegiline, restore dopaminergic tone in rhesus monkeys, however, it also results in enhanced viral replication and brain lesions (Czub et al., 2001). Indeed, *in vitro* studies reveal that cocaine enhances viral replication in monocytes (Peterson et al., 1991), macrophages (Roth et al., 2002), and astrocytes (Reynolds et al., 2006). Cocaine can also enhance monocyte migration across the blood brain barrier and disrupt intercellular junctions (Fiala et al., 1998; Fiala et al., 2005), potentially allowing greater infectivity of CNS cells and exposing CNS cells to potentially neurotoxic molecules that would have previously been excluded by an intact blood-brain barrier. Together, these pre-clinical studies reveal that the enhancement of neuronal damage *in vivo* by cocaine is most likely

a combination of interactions with neurotoxic proteins and increased presence of infected cells, resulting in dire consequences in HIV-1+ patients.

Pre-clinical and clinical studies have attempted to elucidate the mechanism underlying cocaine + HIV-1 neurocognitive deficits, in hopes to reveal a therapeutic target. Cocaine potentiates the neurotoxicity of HIV-1 proteins *in vitro* (Nath, Maragos, Avison, Schmitt, & Berger, 2001; Turchan et al., 2001; Aksenov et al., 2006). HIV-1+ individuals with cognitive deficits have fewer DATs regardless of cocaine use, although there is a trend for cocaine users to have lower DAT levels in the nucleus accumbens and basal ganglia (Chang et al., 2008; Wang et al., 2004). As previously discussed in section 1.1, the dopaminergic system is particularly sensitive to HIV-1, and increased damage to this system is correlated with the severity of neurocognitive deficits (Purohit et al., 2011; Berger & Nath, 1997; Chang et al., 2008; Kumar, Ownby, Waldrop-Valverde, Fernandez, & Kumar, 2011; Nath et al., 2000). However, results from a CHARTER study indicated that past drug use was not predictive of neurocognitive impairment (Byrd et al., 2011), suggesting that enhanced neuronal injury induced by illicit drugs is reversible if abstinence is achieved.

Estrogen and Phytoestrogens: Implications for HIV-1 and Cocaine Use

Flavonoids are secondary metabolites of plant compounds containing a common phenyl benzopyrone feature (Harborne & Williams, 2000). Frequently found in medicinal plants, flavonoids originally caught the attention of researchers due to their natural affinity for $GABA_A$ receptors, specifically the benzodiazepine binding site, and the potential anxiolytic properties mediated at this site (Marder & Paladini, 2002). The flavonoid family includes six major subclasses: flavonols, flavones, isoflavones,

catechins or flavanols, flavanones, and anthocyanidins. These compounds share a common backbone system with varying degrees of oxidation of the pyran ring (Marder & Paladini, 2002), and movement of the B-ring to carbon 2 of the 3-carbon chain to form the isoflavonoids (Romano et al., 2013). Flavonoids are polyphenolic compounds that are frequently found attached to glycosides (sugars), making the compounds water soluble (Ross & Kasum, 2002).

The soy derived compounds genistein, daidzein, the daidzein metabolite equol, and a compound derived from Chinese licorice root, liquiritigenin, are found in the isoflavone class of the flavonoids, and are more specifically identified as phytoestrogens. Unlike the majority of flavones and isoflavones, which were first noticed for their affinities for the benzodiazepine receptor site, phytoestrogens have an affinity for estrogen receptors. There are three subtypes of the estrogen receptor: alpha (ERα), beta (ERβ), and G protein-coupled receptor-30 (GPR-30). ERα and ERβ are classic examples of steroid receptors; they are found intracellularly where the estrogen-ER complex binds to the estrogen response elements (ERE), the general transcription factors that transcribe estrogen responsive genes (Marino, Galluzzo, & Ascenzi, 2006). GPR-30 is a membrane associated receptor thought to be responsible for the more immediate effects of estrogen (Soltysik & Czekaj, 2013). Similar to other flavonoid compounds, genestein and daidzein have been found to be successful in treating various types of cancer, cardiovascular diseases, and osteoporosis (Havsteen, 2002; Jackson, Greiwe, & Schwen, 2011).

Estrogen suppresses HIV-1 replication at the transcriptional level through modulation of the Tat-LTR interaction in glial cells, and this mechanism is not mediated through ERα (Wilson et al., 2006). Conversely, over expression of ERα in glial cells

precludes estrogen mediated transcription suppression (Heron, Turchan-Cholewo, Bruce-Keller, & Wilson, 2009), and ERα may actually increase HIV-1 transcription in the presence of estrogen (Katagiri, Hayashi, Victoriano, Okamoto, & Onozaki, 2006). Meanwhile, activation of ERβ does not increase HIV-1 transcription (Katagiri et al., 2006), however it is unclear whether ERβ plays a role in the estrogen mediated suppression of HIV-1 transcription in glial cells.

Phytoestrogens have a higher affinity for the beta subtype of the estrogen receptor. Relative to 17β-Estradiol genestein has a relative binding affinity for ERβ of 6.8%, with 324 fold selectivity for ERβ over ERα, while daidzein has a lower relative binding affinity of 0.051% for ERβ with 17-fold selectivity for ERβ over ERα. Daidzein is metabolized in the gut to form the more potent metabolite equol; Equol has a 1.52% relative binding affinity compared to 17β-estradiol for ERβ, and a 24-fold selectivity for ERβ over ERα (Jiang et al., 2013a). Liquiritigenin does not preferentially bind to ERα or ERβ, but only initiates transcription through ERβ (Paruthiyil et al., 2009; Mersereau et al., 2008). The ERβ specificity of these compounds indicates that they may provide neuroprotection and more importantly suppress HIV-1 transcription.

ERβ is found in the brain with differing regional densities. The distribution of the ER nuclear receptors α and β in the midbrain has yielded different, and often opposing, results. Although it is generally accepted that ERα and ERβ are found throughout the brain, the localization within regions of the midbrain remain unclear. Zhou and colleagues (Zhou, Cunningham, & Thomas, 2002a) found little to no signal for either α or β mRNA in the VTA of ovariectomized (OVX) female rats, and unquantifiable amounts of ERβ transcript in the midbrain. On the other hand, an earlier study found the exclusive

expression of ERβ mRNA in the VTA of ovariectomized (OVX) female rats (Shughrue, Lane, & Merchenthaler, 1997a). Another study found ERβ protein was co-localized with TH and located in a small population of neurons in the rostral portion of the VTA (Creutz & Kritzer, 2002a), suggesting the ERβ may modulate the dopaminergic system.

There are few studies examining the effects of the different estrogen receptor subtypes on any stage of addiction, and the studies that do exist provide a conflicting picture. In one of the few studies examining the precise role of estrogen receptors in cocaine reinstatement, rats treated with an ERβ agonist prior to drug-induced reinstatement responded significantly more on a lever previously paired with cocaine (Larson & Carroll, 2007a), suggesting the ERβ plays an important role in drug-primed reinstatement. However, another study found that ERβ agonists inhibited ACTH and corticosterone release in response to stress (Weiser, Foradori, & Handa, 2008); suggesting that stress induced reinstatement of cocaine seeking behavior, in which corticosterone plays a major role (Goeders, 2002), may be prevented or attenuated by a selective ERβ agonist, like the phytoestrogens. These studies highlight the importance of understanding the role receptor subtypes play in addiction.

Estrogen may interact with the dopaminergic system through a variety of ways – however, the roles of the relatively newly described estrogen receptor, GPR-30, in addiction and modulation of the dopaminergic system are ill-defined. Estradiol increases dopamine release in the striatum following amphetamine treatment (Xiao & Becker, 1998), suggesting the involvement of estrogen receptors in stimulant effects. Surprisingly, this effect was still present following conjugation of estradiol with BSA (Xiao & Becker, 1998), which would prevent the steroidal hormone from crossing the

cell membrane and interacting with either nuclear ERα or ERβ, indicating an interaction at the membrane level. As previously described GPR-30 is a newly classified membrane estrogen receptor and has been found in the substantia nigra of the striatum (Brailoiu et al., 2007). Thus, suggesting that estrogen and the phytoestrogens may act to enhance dopamine release during exposure to stimulants via the GPR-30, and not ERα or ERβ.

Flavonoids and isoflavonoids are compounds found in an abundance of natural products. These compounds have novel therapeutic value, including antiviral and anti-inflammatory properties (Ding, 1987; Harborne & Williams, 2000; Vrijsen et al., 1988). A recent study determined that the flavonoid luteolin had anti-HIV-1 properties; specifically through preventing activity at the Tat-LTR interaction site, preventing transcription (Mehla, Bivalkar-Mehla, & Chauhan, 2011). ERβ selectivity may prevent unwanted side effects associated with non-selective treatments, and may also minimize the negative effects of ERα activation on Tat-HIV-1 LTR interactions.

HIV-1 Transgenic Rat

The HIV-1 Transgenic Rat (HIV-1 Tg) was developed almost 15 years ago (Reid et al., 2001). This small animal model of HIV-1 includes 7 of the 9 HIV-1 genes, with *gag* and *pol* deleted. The functional deletion of *gag* and *pol*, genes responsible for structural integrity of the virus (Nath, 2002; Pavlakis & Felber, 1990), eliminates the ability of the virus to become infectious or produce an efficient virus. Although this viral construct has been inserted into the mouse, highest viral expression was found in the muscle and skin of the mouse (Dickie et al., 1991). Mouse cyclin T, an important co-factor for LTR-Tat interaction, does not interact functionally with Tat thus preventing transcription initiation (Wei, Garber, Fang, Fischer, & Jones, 1998). Unlike this mouse

model, the viral promoter is fully functional in the HIV-1 Tg rat as evidenced by the presence of spliced and unspliced viral transcripts in the brain, lymph nodes, thymus, liver, kidney, and spleen (Reid et al., 2001; Peng et al., 2010). The active viral promoter and production of HIV-1 viral proteins makes this an excellent model of HIV-1+ individuals with viral suppression.

Initial reports of the HIV-1 Tg rat detail the development of mild to highly opaque cataracts, and the development of AIDS-like pathology (skin lesions, wasting, neurological abnormalities, and respiratory difficulty) within 5-9 months of age (Reid et al., 2001). Later reports using the now commercially available HIV-1 Tg rat describe a model that is relatively healthy for up to 11 months of age, however there was a shift of mRNA expression from the periphery to the CNS in young vs older animals (Peng et al., 2010). The shift in viral expression suggests more severe neurocognitive deficits with age, which is similar to what is seen in humans with HAND (Wiley et al., 1998; Marquine et al., 2014).

The HIV-1 Tg rat displays spatial learning and memory deficits (Lashomb, Vigorito, & Chang, 2009; Vigorito, Lashomb, & Chang, 2007), deficits in executive function (Moran, Booze, & Mactutus, 2014), alterations in sensorimotor gating (Moran, Booze, & Mactutus, 2013), and alterations in behavioral responses following methamphetamine challenge (Moran, Aksenov, Booze, Webb, & Mactutus, 2012; Liu et al., 2009). Neuropathological indicators in the HIV-1 Tg rat are also similar to humans with HAND. The HIV-1 Tg rat has alterations in dendritic spines on the medium spiny neurons in the nucleus accumbens (NAc), lower dopamine binding in the ventral and dorsal striatum, decreased striatal volume, decreased levels of tyrosine hydroxylase (Lee

et al., 2014), and altered sensitivity to cocaine (McIntosh, Sexton, Pattison, Childers, & Hemby, 2015). Methamphetamine challenged HIV-1 Tg rats not only had decreased levels of TH, but also an increase in monoamine oxidase-A (MAO-A) levels (Moran et al., 2012), suggesting a potential compensation mechanism in the HIV-1 Tg rat in response to increased dopamine release due to methamphetamine exposure.

Experimental Goals and Hypotheses

The experimental hypotheses of this dissertation involve both behavioral and neurochemical questions:

1. (Chapters 2-4) Since synaptic loss occurs before cell death and HIV-1 proteins and cocaine result in elevated levels of cell death, do HIV-1 proteins interact with cocaine to increase neuronal damage? Can this damage be attenuated or reversed with the use of phytoestrogen compounds? What is the underlying mechanism of phytoestrogen treatment *in vitro*?

Hypothesis: HIV-1 Tat and cocaine will produce enhanced levels of synaptodendritic damage and treatment with phytoestrogens will prevent and restore synaptic loss. Phytoestrogenic effects will be mediated through an estrogen receptor mediated mechanism.

2. (Chapter 5) HIV-1 produces extensive damage throughout the dopaminergic system of both HIV-1+ humans and HIV-1 Tg rats, a key regulator of motivated behavior. HIV-1+ humans display increased levels of apathy, do HIV-1 Tg rats also display altered motivational processes for natural and/or drug reinforcement? Is dopamine regulation altered following cocaine self-administration?

Hypothesis: The HIV-1 Tg rat will have an altered response to sucrose and cocaine. Dopamine transporter function will be significantly altered by self-administration, and the HIV-1 Tg rat will have a dysfunctional dopamine transporter relative to F344 controls.

3. (Chapter 6) Can ingestion of dietary phytoestrogens attenuate or normalize altered motivation in the HIV-1 Tg rat? Does equol alter morphological characteristics of the medium spiny neurons in the nucleus accumbens?

Hypothesis: Treatment with the phytoestrogen S-equol will normalize the internal motivational state of the HIV-1 Tg rat for sucrose and cocaine. S-equol will have long-lasting effects on behavior and neuromorphological characteristics.

CHAPTER 2

HIV-1 TAT PROTEIN VARIANTS: CRITICAL ROLE FOR THE CYSTEINE REGION IN SYNAPTODENDRITIC INJURY[1]

[1] SJ Bertrand, MV Aksenova, CF Mactutus, RM Booze. 2013. *Experimental Neurology*.248: 228-235.

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Introduction

Human immunodeficiency virus (HIV)-1 infects up to 34 million people worldwide (World Health Organization, 2011). HIV-1 enters the central nervous system early in infection (Nath, 2002; Resnick et al., 1988), where it has been shown to be present in microglia and in a small percentage of astrocytes (An et al., 1999; Ellis et al., 2007; Gonzalez-Scarano & Martin-Garcia, 2005). HIV-1 infection of the central nervous system result in a spectrum of neurocognitive deficits referred to as HIV-1 associated neurocognitive disorders (HAND) (Antinori et al., 2007). The incidence of the most severe form of HAND (i.e., HIV-1 associated dementia), has decreased since the implementation of combination anti-retroviral therapy (cART); nevertheless, neurocognitive impairments still affect 40%-70% of HIV-1 infected individuals (Heaton et al., 2011; Letendre, 2011; Lindl et al., 2010).

The HIV genome consists of three major coding regions: core (gag); polymerase (pol); and envelope (env). Regulatory proteins, such as Tat, play a key role in the pathogenesis of HIV infection and are also encoded in the HIV genome (Pavlakis & Felber, 1990). The HIV-1 transactivator of transcription (Tat) stabilizes the transcription process in order to more efficiently replicate the virus (Dayton et al., 1986; Debaisieux et al., 2012; Gatignol & Jeang, 2000; Pugliese et al., 2005). Tat protein is encoded by two exons with several well-conserved protein domains found on the first exon: the acidic domain (1-21), the cysteine rich domain (22-37), the core/hydrophobic region (38-48), and the basic domain (49-72). Mutations of deletions in residues 22-40 of the first exon

are deleterious to transactivation function, whereas modifications are relatively well tolerated within the acidic domain (1-21) and within the glutamine rich region (58-72) found in the basic domain, thereby highlighting the importance of the different domains of the HIV-1 Tat protein (Jeang, Xiao, & Rich, 1999; Kalantari et al., 2008; Kuppuswamy, Subramanian, Srinivasan, & Chinnadurai, 1989; Ruben et al., 1989). More investigations examining the biological effects of Tat protein functional domains have focused on the transactivational ability of the protein (Debaisieux et al., 2012; Hauber, Malim, & Cullen, 1989; Kalantari et al., 2008; Kubota, Endo, Maki, & Hatanaka, 1989; Kuppuswamy et al., 1989; Li et al., 2012).

HIV-1 does not directly infect neurons (Gonzalez-Scarano & Martin-Garcia, 2005; Nath, 2002). However, HIV-1 Tat is released into the extracellular space of the brain (King, Eugenin, Buckner, & Berman, 2006; Nath, 2002; Pugliese et al., 2005). Elevated levels of *Tat* mRNA have been found in the brain tissue of individuals with HAD and HIV-1 encephalitis (Cowley, Gray, Wesselingh, Gorry, & Churchill, 2011; Hudson et al., 2000; Wesselingh et al., 1993; Wiley, Baldwin, & Achim, 1996). HIV-1 Tat has also been detected via immunostaining of brain tissue in individuals with HIV-1 encephalitis (Del et al., 2000; Hudson et al., 2000; Kruman et al., 1999; Liu et al., 2000), and can also be detected in the brains of rhesus macqaques infected with a HIV/SIV chimera (Hudson et al., 2000; Kruman et al., 1999). HIV-1 Tat has been shown to initiate neuronal cell death *in vitro* (Adams, Aksenova, Aksenov, Mactutus, & Booze, 2010; Adams et al., 2012; Aksenov, Aksenova, Mactutus, & Booze, 2009; Aksenova et al., 2006; Bonavia et al., 2001) and *in vivo* (Aksenov et al., 2003; Aksenov et al., 2001; Bansal et al., 2000; Fitting, Booze, & Mactutus, 2008; Fitting, Booze, Hasselrot, &

Mactutus, 2008; Fitting, Booze, Hasselrot, & Mactutus, 2010; Kim et al., 2003; Wang, Barks, & Silverstein, 1999). Neuronal cell death has been reported in post-mortem HIV-1 brain tissue (Adle-Biassette et al., 1995; Adle-Biassette et al., 1999); however, cell death correlates poorly with neurocognitive status (Adle-Biassette et al., 1999; Kaul, Garden, & Lipton, 2001).

In contrast to cell death, dendritic pruning, synaptic loss, and dendritic simplification correlate well with HAND (Adle-Biassette et al., 1999; Everall et al., 1999; Kaul et al., 2001). Synaptic injury may reflect early, sometimes asymptomatic, stages of HAND, before extensive neuronal loss occurs (Masliah et al., 1997). The HIV-1 Tat protein has been reported to cause a loss in synaptic density, dendritic pruning, and dendritic simplification both *in vivo* (Fitting et al., 2010a) as well as *in vitro* (Kim et al., 2008a). Although the role of the functional domains of Tat has been explored in regard to neuronal cell death (Aksenov et al., 2009; Mishra, Vetrivel, Siddappa, Ranga, & Seth, 2008), the role of the Tat protein functional domains in producing early synaptodendritic damage is poorly understood.

Filamentous actin (F-actin) is a major cytoskeletal protein that makes up both pre- and post-synaptic structures (Bleckert, Photowala, & Alford, 2012; Cingolani & Goda, 2008), and F-actin is a key target for stabilizing or destabilizing cellular signals which ultimately produce stable synapses (Zhang & Benson, 2001). In this process, globular acting (G-actin) is polymerized into F-actin, a more stable form of actin (Hotulainen et al., 2009; Johnson & Ouimet, 2006; Sekino, Kojima, & Shirao, 2007). Although dendritic spines are some of the most well-studied F-actin rich structures, F-actin is a component of a variety of neural structures. For example, F-actin is a major cytoskeletal protein in

filopodia (long, headless structures or "pre spines") (Hotulainen et al., 2009; Jacinto & Wolpert, 2001; Kaech, Parmar, Roelandse, Bornmann, & Matus, 2001; Sekino et al., 2007), and patches or "hot spots" along the dendrite appear as F-actin rich puncta (Halpain, Hipolito, & Saffer, 1998). F-actin rich patches appear both at the initiation of spinogenesis (Johnson & Ouimet, 2006), as well as in non-spiny synapses, which are typically GABA-ergic (Craig, Blackstone, Huganir, & Banker, 1994; van Spronsen & Hoogenraad, 2010). Phalloidin, a form of phallotoxin isolated from the death cap mushroom (*Amanita phalloides*) selectively binds to F-actin, but not monomeric G-actin, and has been used to examine the dynamic activity of F-actin in modulating synaptic changes (Halpain et al., 1998; Hotulainen et al., 2009; Kaech, Fischer, Doll, & Matus, 1997; Korobova & Svitkina, 2010). In the present study, the term F-actin puncta referes to filopodia, patches, and protruding spines but not growth cones (F-actin rich structurese at the most distal ends of the neurite). Quantification of F-actin puncta detects pre- and post-synaptic changes as awell as changes in the number of inhibitory and excitatory synapses. Thus, increases or decreases in the quantity of F-actin puncta suggest disruptions in excitatory and inhibitory signaling, which may translate to dysfunctional synaptic communication.

Previous work in our laboratory has shown that neuronal death *in vitro* occurs after 48 hours of exposure to Tat 1-86 (Aksenov et al., 2009), and moreover, a mutation at Cys22 in Tat 1-86 eliminates neuronal death induced by HIV-1 Tat 1-86 exposure (Aksenov et al., 2009). The present study examines early Tat protein effects on dendritic integrity at a time prior to cell death, and additionally uses synthetic HIV-1 Tat peptides to examine the importance of the highly conserved cysteine rich domain in producing

synaptodendritic injury, an important indication of function in HAND. Full length Tat 1-101, Clades B and C, was used to examine specific genetic differences of the Tat protein in producing early synaptic damage which may reflect differences in neurocognitive impairments between Clades B and C. Synthetic peptide lengths 1-72, 1-72δ31-61, 1-86, 1-86[Cys22] and 47-57 were used to partition the capacity of the various Tat protein functional domains for inducing synaptodendritic damage. Our results suggest that synaptic injury due to Tat exposure occurs prior to cell death and is dependent upon key protein residues.

Methods

Primary hippocampal cell cultures. Primary hippocampal cell cultures were prepared from 18-day old Sprague-Dawley rat fetuses as previously described (Aksenov et al., 2009; Aksenov et al., 2006; Bertrand, Aksenova, Aksenov, Mactutus, & Booze, 2011). Procedures were carried out in accordance with the University of South Carolina Institutional Animal Care and Use Committee (animal assurance number: A3049-01). Rat hippocampi were dissected and incubated for 10 minutes in a solution of 2mg/ml trypsin in Hank's balanced salt solution (HBSS) buffered with 10 mM HEPES (GIBCO Life Technologies, Grand Island, NY). The tissue was then exposed for 2 minutes to soybean trypsin inhibitor (1mg/ml in HBSS) and rinsed 3 times in HBSS. Cells were dissociated by trituration and distributed to poly-L-lysine coated culture dishes (Costar, Cambridge, MA; MatTek Corporation, Ashland, MA). For cytomorphological studies, hippocampal cells were cultured in 35mm glass-bottom cell culture dishes (MatTek Corporation, Ashland, MA). For cell viability measurements, hippocampal cell cultures were grown in 96-well plastic plates (Costar, Cambridge, MA). At the time of plating, plates contained

DMEM/F12 (GIBCO) supplemented with 100 mL/L fetal bovine serum (Sgima Chemicals, St. Louis, MO). After a 24-h period, DMEM/F12 was replaced with an equal amount of serum-free Neurobasal medium supplemented with 2% v/v B-27, 2mM GlutaMAX supplement and 0.5% w/v D-(1)-glucose (all ingredients from GIBCO). Cultures (maintained in the serum-free growth medium) were used for experiments at the age of 14-21 days in vitro (DIV) and were >85-90% neuronal as determined by anti0MAP-2/anti-GFAP/Hoescht fluorescent staining.

Experimental treatment of cultures. The treatment of hippocampal cell cultures with HIV-1 Tat was carried out by the addition of 10µL of freshly prepared stock solution of a recombinant Tat variant (Table 1.1) into the cell culture growth medium. An equal volume of vehicle was added to control cell cultures. Tat variants were produced using similar recombinant and purification techniques and were determined to be greater than 90% pure and free of endotoxin contamination. Primary hippocampal cells were incubated with 50nM of each Tat variant for either 24 h (F-actin) or 48 h (Live/Dead viability). Investigators were blind to the treatment condition of the cultures.

Table 2.1 List of HIV-1 Tat variants, lengths, and vendors.

Peptide Length	Clade	Vendor
1-86	B	Diatheva, Italy
1-86[Cys22]	--	Diatheva, Italy
1-101	B	Immunodiagnostic, MA USA
1-101	C	Prospec, Israel
1-72δ31-61	--	U. of Kentucky, USA
1-72	B	U. of Kentucky, USA
47-57	--	Anaspec, CA USA

Fluorescent labeling and immunocytochemistry – 24 h Tat exposure. The fluorescent and immunofluorescent labeling of hippocampal cells were carried out in

primary cultures prepared in 35 mm glass-bottom cell culture dishes (MatTek Corporation, Ashland, MA). Cultures were fixed with 4% paraformaldehyde and cells were permeabilized in 0.1% Triton X-100 and then incubated for 20 minutes at room temperature with an F-actin specific probe, Alexa Fluor 488 phalloidin (0.165µM) (Invitrogen Life Technologies, Grand Island, NY). Fixed hippocampal cell cultures were incubated with 10% normal horse serum (NHS) in PBS to block nonspecific binding and then used for labeling the cells with rabbit polyclonal MAP2 (1:1000, Santa Cruz Biotechnologiy, Inc, Santa Cruz,CA). The secondary antibody for immunofluorescent staining was Alex Red 594-conjugated goat anti-rabbit IgG (1:5--, Invitrogen Life Technologies, Grand Island, NY). Blue fluorescent Hoescht dye was used to identify cell nuclei.

Images of F-actin/MAP2 fluorescently co-labeled hippocampal cell cultures were acquired using a high resolution CCD camera attached to the Nikon Eclipse TE2000-E inverted fluorescent computer-controlled microscope (20x objective, 1600 x 2000 pixel image size, 0.17 µm/px image resolution at 1x zoom). At least 3 [Green (F-actin; phalloidin staining)/Red (MAP2 immunolabeling)/Blue (Hoescht staining)] individual pyramidal neurons with clearly defined dendritic arbors and normal nuclear morphology per culture well were randomly selected and analyzed using the NIS-Elements software package (Nikon). For each neuron selected, F-actin rich synaptic structures were manually counted in several (2-3) digitally magnified dendrites (25-75µm-long, second order bifurcations). Only those dendritic segments with normal morphology (continuous MAP-2 staining; no beading or varicosities) were included in the analysis. F-actin puncta were identified by using the 518 wavelength emission channel. The low background

fluorescence of non-synaptic structures was subtracted (20-60 fluorescence units), allowing clear visualization of puncta. The length of the neurite segment was recorded and the number of puncta was divided by this length and multiplied by 10 yielding puncta per 10 µm. F-actin rich structures, or puncta, included for analysis were fine filopodia, spine protrusions, and dendritic F-actin patches. Growth cones (F-actin rich structures located at the most distal dendritic terminus) were excluded from analysis.

Cell viability – 48 h Tat exposure. The hippocampal cell viability was assessed in primary neuronal cell cultures prepared in 96-well cell culture plates (Costar, Cambridge, MA) using the microplate reader-formatted variant of the fluorescent calcein AM/ethidium bromide cell labeling assay (Live/Dead kit, Invitrogen, Carlsbad, CA) as described previously (Adams et al., 2010; Adams et al., 2012; Aksenova et al., 2009; Aksenov et al., 2006; Aksenova et al., 2006). Fluorescence was measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Data are presented in a ratio of calcein fluorescence (live) divided by ethidium fluorescence (dead).

Statistical analysis. Statistical comparisons were performed with SPSS version 19 (IBM, New York, NY) using a two-way ANOVA. Tukey's multiple comparison tests were used to determine specific treatment effects. Significant differences were determined with an alpha level set at $p \leq 0.05$. Data are presented as mean values ± standard error of the mean (SEM).

Results

Tat 1-86B and Tat 1-101B produce similar synaptodendritic damage.

Tat 1-86B and Tat 1-101B are both Clade B protein variants differing only in their residue length. The current experiment was performed to evaluate differences, if any, in puncta loss, cell viability and neurite integrity between the two variant lengths. The synaptodendritic integrity of primary hippocampal cells was evaluated by calculating the density of F-actin puncta 24 h after exposure to Tat 1-101B (50nM) or Tat 1-86 (50nM). There was an overall significant main effect of treatment on puncta loss $F(2,24)=13.14$, ($p \leq 0.001$). Tukey's multiple comparison tests confirmed that Tat 1-101B ($p \leq 0.001$) and Tat 1-86 ($p \leq 0.001$) induced significant loss of F-actin puncta when compared to controls, but failed to differ significantly from one another (Fig 2.1A).

Cell viability of primary hippocampal cell cultures was evaluated 48 h after exposure to 50nM Tat 1-86B or 50nM Tat 1-101B using a Live/Dead assay. There was an overall significant main effect of treatment on viability $F(2.24)=70.24$, ($p \leq 0.001$). Post-hoc analyses using Tukey's multiple comparisons test determined that Tat 1-101B ($p \leq 0.001$) and Tat 1-86 ($p \leq 0.001$) produced significant cell death, 34% and 29% respectively, when compared to controls, but failed to differ significantly from one another (Fig 2.1B).

After 24 h, neurons displayed a normal pyramidal morphology (Fig 2.1C). However, relative to control neurons, which had robust F-actin staining and complex dendritic branching patterns, both Tat 1-86B and Tat 1-101B treated neurons demonstrated decreased branching, decreased F-actin and an overall simplification of the neuronal network.

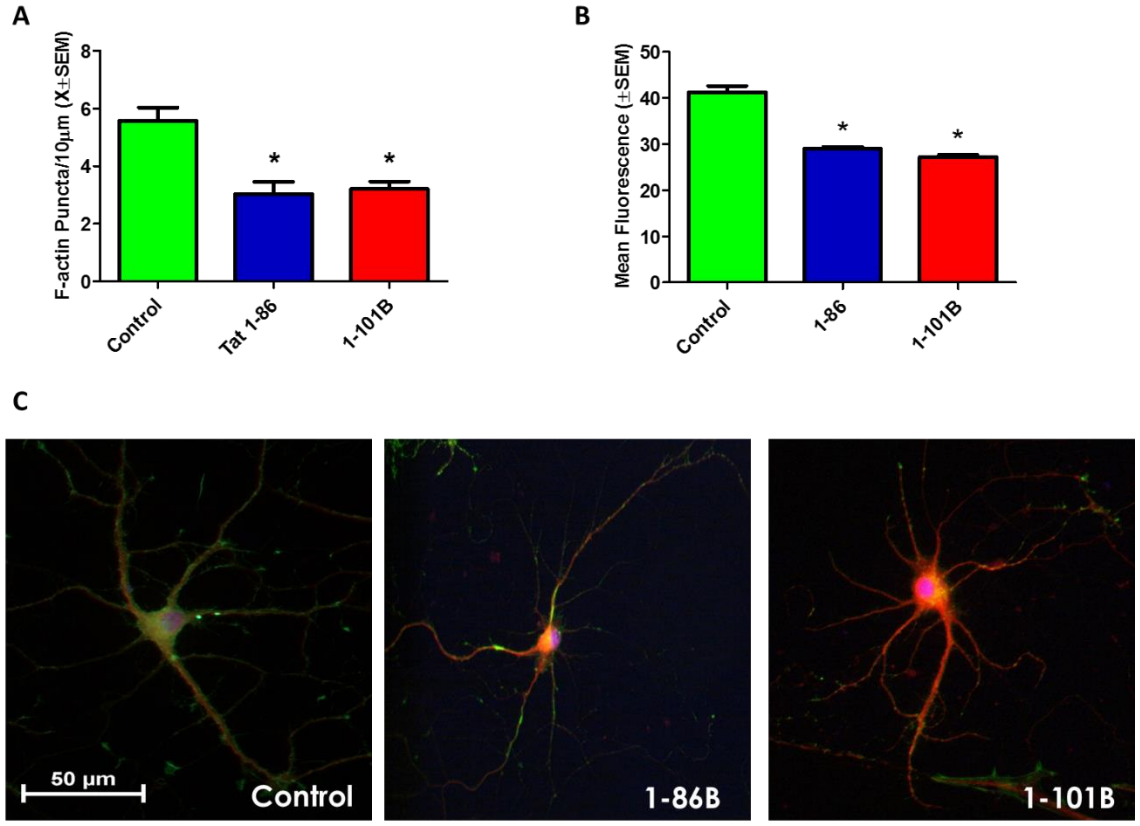

Figure 2.1 Tat 1-86 and Tat 1-101B produce similar synaptodendritic damage. **A.** Tat 1-86 or Tat 1-101B treatment (50nM) for 24 h produced significant loss of F-actin puncta. Second order dendritic branches were selected from 20x images of F-actin/MAP2/Hoescht-stained Tat-treated control pyramidal hippocampal neurons for puncta quantification. Results are presented as mean F-actin labeled puncta per 10 µm of neuronal dendrite ± SEM. *-- indicates a significant ($p \leq 0.05$) difference relative to control. **B.** Tat 1-86 and Tat 1-101B treatment (50nM) for 48 hours produced significant cell death. Fluorescent units were determined in hippocampal cell cultures using Live/Dead assays. Results are presented as mean fluorescence values ± SEM. *--indicates a significant ($p \leq 0.05$) difference relative to control. **C.** Contronl Tat 1-86 (50nM) and Tat 1-101B (50nM) treated neurons labeled with F-actin (green)/MAP-2 (red)/Hoescht (blue. The hippocampal pyramidal neuron in the control condition demonstrated robust F-actin labeling (green) and complex branching patterns. Both of the Tat treated neurons had greatly diminished F-actin staining (green), and decreased dendritic branching (red) relative to controls. Scale bar = 50µm.

Tat 1-86[Cys22] and Tat 1-101C do not produce damage.

Tat 1-101C is a naturally occurring variant of the Tat protein with a mutation at Cys31,

whereas Tat 1-86[Cys22] is a synthetic peptide with a mutation at Cys22. Our previous

work demonstrated that a mutation in the cysteine rich domain attenuates decreases in cell viability at 48h after Tat 1-86 exposure (Aksenov et al., 2009). The present experiment was undertaken to determine if changes in puncta loss, viability or neuronal morphology were residue specific, or could be generalized to the cysteine rich domain. The synaptodendritic integrity of primary hippocampal cells was evaluated by calculating the density of F-actin puncta 24h after exposure to either Tat 1-101C (50nM) or Tat 1-86[Cys22] (50nM). There was no significant overall main effect of treatment on F-actin puncta (Fig 2.2A). Cell viability of primary hippocampal cell cultures was evaluated 48 h after exposure to 50nM Tat 1-101C or 50nM Tat 1-86[Cys22] produced neuronal cell death (Fig 2.2B). The morphologic appearance of the hippocampal pyramidal cells (Fig 2.2C) did not differ from controls with all groups displaying intact dendrites, complex branching patterns and abundant F-actin staining.

The cysteine rich region of Tat is critical for synaptodendritic damage.

The prior experiments determined that the cysteine region was vital to the loss of F-actin puncta and cell death. This experiment examined the effects of the cysteine region relative to the core and basic domains of the Tat protein. Tat 1-72 was used to determine if the second exon was essential for either the puncta loss, cell death, or dendritic integrity. The Tat protein deletion mutation, Tat 1-72δ31-61, further assessed the relative importance of the cysteine rich domain and the arginine rich domain. However, as a small portion of the cysteine rich domain was present in the deletion mutant, the short peptide Tat 47-57 was also examined to assess the importance of internalization of the protein to puncta loss and cell death.

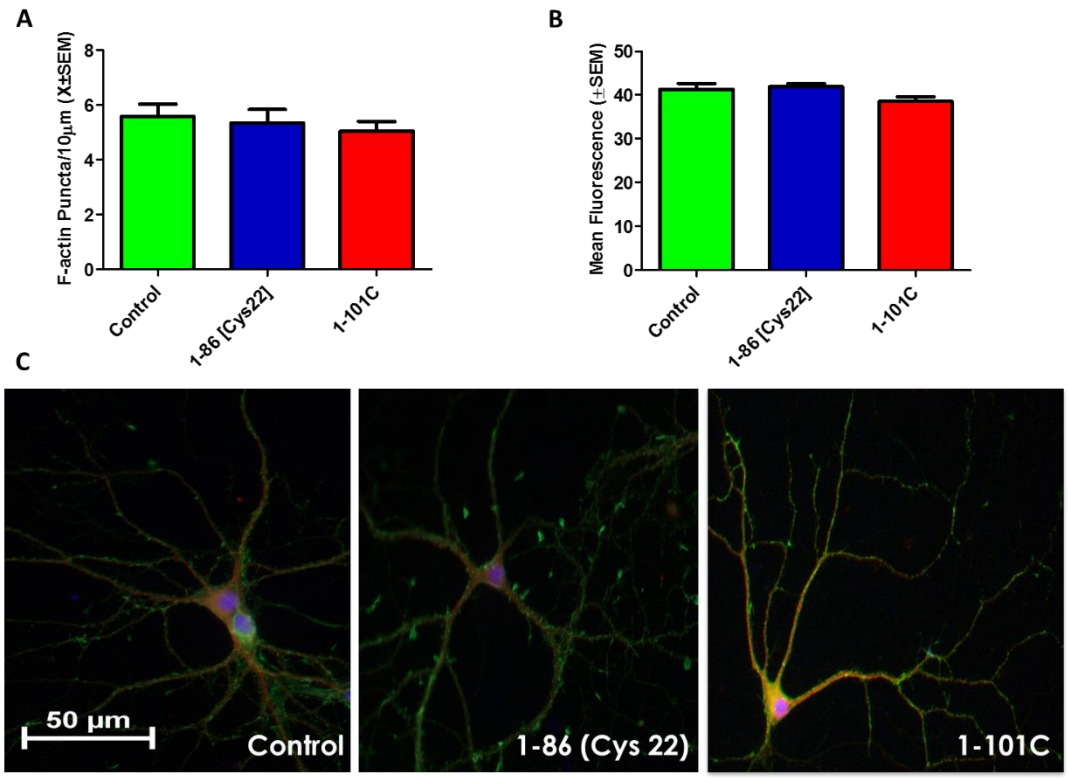

Figure 2.2 Tat 1-101C and Tat 1-86 (Cys22) do not produce synaptodendritic injury. **A.** Tat 1-86 (Cys22) or Tat 1-101 Clade C treatment (50nM) for 24 h did not produce significant loss of F-actin puncta relative to controls. Second order dendritic branches were selected from 20x images of F-actin/MAP2/Hoescht-stained Tat-treated and non-treated control pyramidal hippocampal neurons for puncta quantification. Results are presented as mean F-actin labeled puncta per 10 µm of neuronal dendrite ±SEM. **B.** Tat 1-101 Clade C or Tat 1-86 (Cys22) treatment (50nM) for 48 h did not produce cell death. Fluorescent units were determined in hippocampal cell cultures using Live/Dead assays. Results are presented as mean values ± SEM. **C.** Control, Tat 1-86 (Cys22) (50nM) and Tat 1-101 Clade C (50 nM) treated neurons labeled with F-actin(green)/MAP-2 (red)/Hoescht (blue). The hippocampal pyranmidal neuron in the control condition demonstrates robust F-actin labeling (green), and complex branching patterns, suggesting that treatment with these peptides does not produce significant changes in dendritic integrity. Scale bar = 50µm.

The synaptodendritic integrity of primary hippocampal cells was evaluated by calculating the density of F-actin puncta 24h after exposure to Tat 1-72 (50nM), Tat 1-72δ31-61(50nM), or Tat 47-57 (50nM). There was a significant overall effect of treatment on puncta density $F(3,32)=4.91$ ($p \leq 0.006$). Tukey's multiple comparisons

confirmed that Tat 1-72 produced significant puncta loss ($p \leq 0.05$), but Tat 1-72δ31-61 and Tat 47-57 failed to produce significant puncta loss compared to controls (Fig 2.3A).

The control, Tat 1-72δ31-61, and Tat 47-57 treated pyramidal neurons all displayed complex branching patterns, extensive fine networks and strong F-actin labeling (Fig. 2.3C). In contrast, neurons treated with Tat 1-72 produced less branching, resulting in simplification of the fine network and reduced F-actin staining.

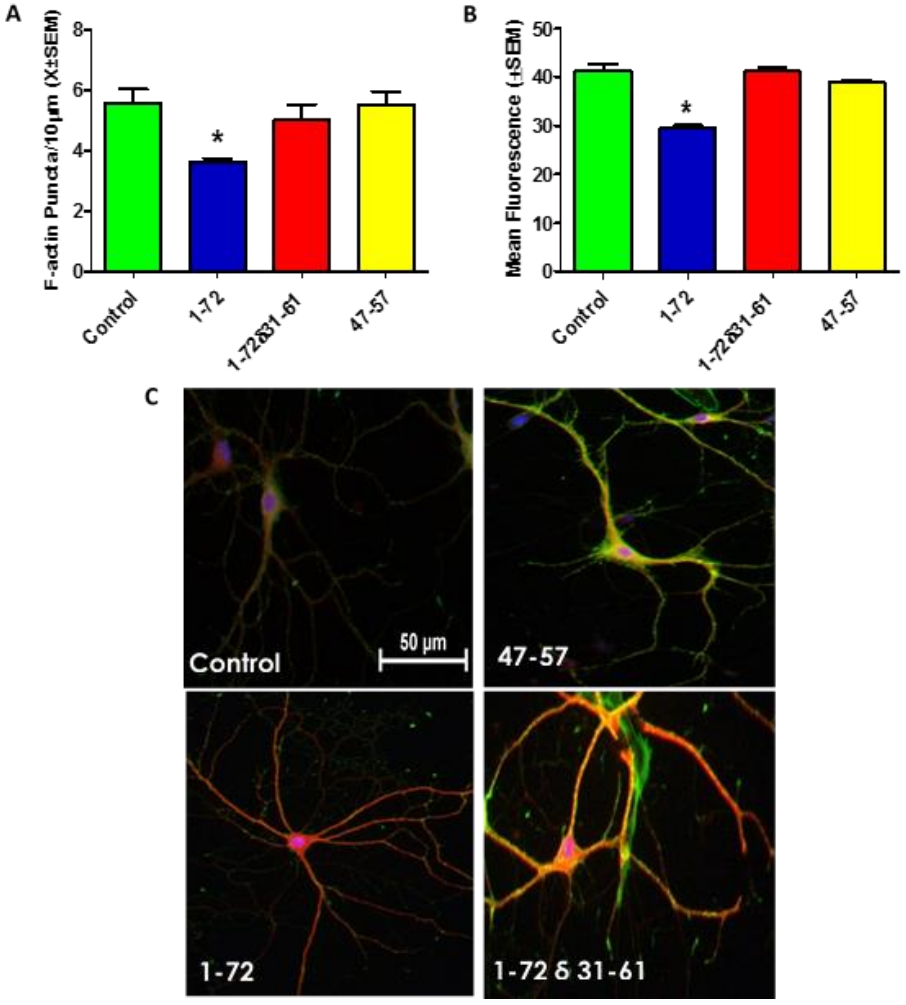

Figure 2.3 The cysteine rich domain of Tat protein is critical for producing synaptodendritic injury. **A.** Tat 1-72 treatment (50nM) for 24 h produced significant loss of puncta relative to controls; however, Tat 1-72δ31-61 (50nM) or Tat 47-57 treatment (50nM) for 24 h did not produce significant loss of F-actin puncta. Second order dendritic branches were selected from 20x images of F-acin/MAP2/Hoescht-stained Tat-treated and non-treated control pyramidal hippocampal neurons for puncta quantification.

Results are presented as mean F-actin labeled puncta per 10 µm of neuronal dendrite ±SEM. *--indicates a significant ($p \leq 0.05$) difference relative to control. **B.** Tat 1-72 treatment (50nM) for 48 h produced significant cell death; however, Tat 1-72δ31-61(50nM) or Tat 47-57 treatment (50nM) for 48h did not produce cell death. Fluorescent units were determined in hippocampal cell cultures using Live/Dead assays. Results are presented as mean values ±SEM. *--indicates a significant ($p \leq 0.05$) difference relative to control. **C.** Control, Tat (47-57) (50nM), Tat 1-72 (50nM), and Tat 1-72δ31-61 (50nM) treated neurons labeled with F-actin (green)/MAP-2 (red)/Hoescht (blue). The hippocampal pyramidal neuron in the control condition, Tat (47-57) and Tat 1-72δ31-61 show robust F-actin labeling (green), and complex branching (red). Treatment with Tat 1-72 resulted in diminished F-actin staining (loss of green) and decreased dendritic branching. Scale bar = 50 µm.

Discussion

In the current study, synthetic HIV-1 Tat peptides of varying lengths with specific mutations or deletions were used to examine the effects of the Tat functional domains on synaptodendritic injury. We found that Tat variants with an intact cysteine rich region (residues 22-37) in the first exon, regardless of protein length, produced significant early damage to the synaptic network and later cell death. Conversely, Tat protein variants with a mutation, deletion, or a lack of a cysteine rich domain, failed to induce synaptic damage or later cell death. These results suggest that the cysteine rich domain plays a role in the early neuropathological event of synaptodendritic injury. Synaptic injury is currently the best neuropathologic correlate of cognitive decline in HAND (Ellis et al., 2007). Synaptic dysfunction and neurocognitive impairments are generally evident long before neurons die in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (van Spronsen & Hoogenraad, 2010; Coleman, Federoff, & Kurlan, 2004). Although the direct relationship between specific synaptic dysfunction(s) and HAND remains unclear, the identification of viral attributes capable of producing synaptic damage/dysfunction (one being the presence of specific Tat protein domains) is key to understanding HAND.

F-actin is a key marker for synapse stability (Zhang & Benson, 2001). Our F-actin stained puncta structurally include excitatory synapses (Hotulainen et al., 2009; Jacinto & Wolpert, 2001), inhibitory synapses (Craig et al., 1994), and both pre-/post-synaptic structures (Bleckert et al., 2012; Cingolani & Goda, 2008), allowing for the overall assessment of synaptic integrity. We observed a reduction of F-actin puncta and general dendritic field simplification in response to Tat peptides with the cysteine rich region intact (Tat 1-72, Tat 1-86, Tat 1-101B). Extending the peptide length (from 72 to 86 and 101 aa) did not significantly affect the pronounced loss of puncta, suggesting that the second exon does not play a key role in Tat-induced synaptodendritic injury. Tat 1-72 is not a naturally occurring human isolate; however, Tat 1-101 (Rosen et al., 1986) and Tat 1-86 (Barre-Sinoussi et al., 1983) have both been reported in human plasma following HIV-1 infection. Although the neural uptake of Tat 1-86 has been shown to be more efficient than that of Tat 1-72 (Ma & Nath, 1997), the present results suggest that protein uptake may not be necessary for puncta loss and dendritic network degradation.

Additionally, Tat 1-72, Tat 1-86, and Tat 1-101B have previously been shown to cause cell death at later time points in cell culture models (Aksenova et al., 2009; King et al., 2006; Mishra et al., 2008). In the present studies we confirmed these original reports for the individual proteins (cell death at 48 h), and further demonstrate that these protein variants do not significantly differ in their ability to decrease viability. Moreover, we now report that these recombinant proteins (1-86[Cys22], 1-101C, 1-72δ31-61), from the same manufacturers and produced under similar conditions, did not produce damage, thereby eliminating the concern of endotoxin contamination confounding the synaptodendritic damage and cell death profile for these particular Tat protein variants.

The Tat protein is subject to genetic variations and modifications (Li et al., 2012). HIV-1 Tat is encoded by two exons, and has several distinct functional domains which are essential for viral replication and are responsible for cell death (Karn, 1999; Kuppuswamy et al., 1989). The first three domains: the acidic domain (1-21), the cysteine rich domain (22-37), and the core/hydrophobic region (38-58) represent the minimal number of residues required for the transactivation capabilities of Tat (Debaisieux et al., 2012; Kuppuswamy et al., 1989). The basic domain (49-72) can be broken down into two subdomains: the arginine-rich domain (49-57) and the glutamine rich domain (58-72) (Li et al., 2012). Modifications and mutations are relatively well-tolerated within the acidic domain (1-21), but mutations in residues 22-40 are deleterious to transactivation function (Jeang et al., 1999; Kalantari et al., 2008; Kuppuswamy et al., 1989; Ruben et al., 1989) and have been shown to attenuate Tat induced neuronal cell death (Aksenov et al., 2009; Campbell, Watkins, Loret, & Spector, 2011).

The present study used peptides with mutations at Cys22 (1-86[Cys22]), and Cys 31 (Tat 1-101C), and demonstrated that these mutations do not cause significant puncta loss. Additionally, we used Tat 47-57 which includes the last two residues of the core domain (38-48) and the arginine rich domain (49-57) – the region responsible for binding to cell membranes and for uptake into adjacent cells (Debaisieux et al., 2012; Jeang et al., 1999; Kuppuswamy et al., 1989; Ruben et al., 1989) – but lacks the cysteine rich domain(22-37), Tat 47-57 did not produce losso f F-actin puncta, suggesting that neuronal uptake aloe is not responsible for Tat effects. Indeed, Tat 1-72δ31-61 (the first exon with the end of the cysteine rich domain, the arginine rich domain, and part of the glutamine rich region deleted), did not produce significant loss of F-actin puncta,

accentuating the importance for the cysteine rich domain for induction of puncta loss, and moreover, that neuronal uptake of the Tat peptide is not necessary for puncta loss.

HIV-1 neurocognitive related deficits have been reported as more common in Clade B prevalent areas (North America and Europe) relative to Clade C areas (Sub-Saharan Africa and Asia) (Satishchandra et al., 2000; however, see Gupta et al., 2007). HIV-1 Tat C, which has a mutation t Cys31, has been shown to be less neurotoxic than Clade B Tat (Aksenov et al., 2009; Mishra et al., 2008; Ranga et al., 2004). The present study confirms that Tat 1-101C also does not produce a significant loss of F-actin puncta. Although Tat may not mediate neurocognitive deficits in the Clade C population, there are other HIV-1 proteins, such as gp120, known to cause neuronal death (Bansal et al., 2000; Kaul et al., 2001) and synapse loss (Kim, Shin, & Thayer, 2011). Thus, elucidating further differences in HIV-1 proteins between the two clades remains an important question and may shed light on the relationship between synaptic dysfunction and HAND.

Conclusions

In the present study, F-actin puncta provided a broad assessment of early synaptodendritic damage produced by variants of HIV-1 Tat protein. An intact cysteine rich domain of the Tat protein exon 1 is critical for producing synaptodendritic injury; however, Tat protein uptake into the neurons is not necessary for damaging synapses. Greater understanding of the cellular mechanisms of synaptodendritic damage and dysfunction may aid in the treatment of HIV-1 assciated neurocognitive disorders. Although the direct relationship between specific excitatory/inhibitory synaptic dysfunction(s) and HAND remains unclear, the identification of viral attributes capable

of producing synaptic damage/dysfunction (e.g. the presence/absence of specific Tat protein alterations) is key to understanding HAND.

CHAPTER 3

SYNAPTODENDRITIC RECOVERY FOLLOWING HIV TAT EXPOSURE:

NEURORESTORATION BY PHTYOESTROGENS[2]

[2]SJ Bertrand, CF Mactutus, MV Aksenova, TD Espensen-Sturges, RM Booze. 2014. *Journal of Neurochemistry*. 218: 140-151.

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Introduction

HIV-1 enters the central nervous system early in the infection process and may ultimately result in an array of deficits, collectively known as HIV-1 associated neurocognitive disorders (HAND) (Antinori et al., 2007). Although the incidence of the most severe dementia has decreased as the development of combination anti-retroviral therapy (cART), neurocognitive deficits continue to persist in more than 40% of HIV-1 infected individuals (Lindl et al., 2010; Letendre, 2011). The continued prevalence of HAND despite the success of cART (Ances & Ellis, 2007; Lindl et al., 2010; Letendre, 2011) indicates a need to understand the underlying mechanism(s) of HAND and identify effective treatments.

Neuronal cell death has been observed in post-mortem brain tissue of patients with HAND; however, cell death does not correlate well with neurocognitive status (Adle-Biassette et al., 1999; Kaul et al., 2001; Ellis et al., 2007). Dendritic pruning, decreases in spine density, and degradation of synaptic proteins all correlate more readily with HIV-1 induced neurocognitive decline than does cell death (Masliah et al., 1997; Adle-Biassette et al., 1999; Kaul et al., 2001). Moreover, loss of dendrites and decreased synaptic density in the frontal cortex from HIV+ patients has been reported with latent viral infection (Desplats et al., 2013).

During latent HIV-1 infection, the provirus is incorporated into cellular DNA, without the expression of viral RNA (Wu, 2004). Current anti-retroviral drugs effectively suppress peripheral viral load, but do not prevent the continued latent

production of HIV-1 neurotoxic proteins (i.e. Tat) once the proviral DNA is incorporated into the brain (Li, Li, Steiner, & Nath, 2009). HIV-1 provirus containing cells can thereby produce and release Tat into the extracellular space (Pugliese et al., 2005; Bachani, Sacktor, McArthur, Nath, & Rumbaugh, 2013) and Tat can interact with the cell surface of other, non-infected cells, such as neurons. HIV-1 Tat protein has been found to decrease dendritic spine and synaptic density *in vitro* and *in vivo* (Everall et al., 1999; Kim et al., 2008a; Fitting et al., 2010a). Therefore, a more detailed understanding of how HIV-1 Tat interacts with the dendritic network may aid in preventing neuropathological dendritic pruning and synaptic loss during latent HIV-1 infection.

Filamentous actin (F-actin) is one of the major cytoskeletal proteins that make up pre- and post-synaptic structures. Polymerization of globular actin (G-actin) into F-actin, a more stable form of actin, occurs prior to spinogenesis (Johnson & Ouimet, 2006). Although dendritic spines are rich in F-actin, where F-actin is found in the spine head and shaft (Sekino et al., 2007; Dent, Merriam, & Hu, 2011), dendritic spines are not the only F-actin rich structures found on the neurite (Hotulainen et al., 2009; Halpain et al., 1998; Lau, Zucker, & Bentley, 1999; Johnson & Ouimet, 2006). F-actin is associated with both pre- and post-synaptic structures (Johnson & Ouimet, 2006), including non-spiny synapses; therefore, changes in the F-actin rich structures i.e. dendritic F-atin puncta, suggest overall alterations in synaptic connectivity not limited to spines. Phalloidin, a form of phallotoxin isolated from the death cap mushroom (*Amanita phalloides*), selectively binds to F-actin, but not monomeric G-actin, and has been used to examine the dynamic activity of F-actin in modulating synaptic plasticity (Kaech et al., 1997; Halpain et al., 1998; Hotulainen et al., 2009; Korobova & Svitkina, 2010).

Interestingly, estrogens modulate spine concentrations of F-actin (Kramar et al., 2009; Sanchez et al., 2009), suggesting estrogenic therapeutic approaches to correcting F-actin loss and restoration of neuroplasticity. Phytoestrogens are plant-derived diphenolic compounds found to partially mimic mammalian estrogen in structure and function (Glazier & Bowman, 2001; Lephart, Setchell, & Lund, 2005). Daidzein (DAI) is a phytoestrogen isoflavone found in soybeans (Mortensen et al., 2009). DAI has been shown to be neuroprotective against glutamate excitotoxicity and $A\beta_{25-35}$ –induced apoptosis (Zhao, Chen, & Diaz, 2002). We have reported that DAI is protective against acute HIV-1 Tat-induced apoptosis (Adams et al., 2012).

The novel flavonoid, liquiritigenin (LQ), is one of the active compounds of MF101, an herbal remedy used to treat menopausal symptoms (Mersereau et al., 2008). LQ has been isolated from Chinese licorice root, *glycyrrhiza uralensis,* and identified asa selective estrogen receptor beta (ERβ) agonist (Paruthiyil et al., 2009). Previous studies have shown that LQ has anti-inflammatory effects, prevents neurocognitive deficits and neurotoxicity induced by the Aβ protein in animal models (Liu, Zou, & Lu, 2009; Liu, Zou, Fu, & Lu, 2010; Liu, Tang, Zou, Fu, & Lu, 2011). LQ has been used in Traditional Chinese Medicine to treat senile dementia (Lin et al., 2012). Together, the ERβ specificity of LQ as well as the ability of LQ to prevent neurocognitive decline in animal models, sugget that LQ could be neuroprotective against HIV-1 Tat-mediated cell death and synaptodendritic damage.

Neurocognitive deficits associated with latent HIV-1 infection continue to persist despite successful suppression of peripheral viral load by anti-retroviral therapy (Heaton et al., 2010; Desplats et al., 2013). Synaptodendritic injury is closely tied to cognitive

decline in HAND (Ellis et al., 2007). However, there are currently no treatments to prevent or attenuate synaptodendritic injury that occurs during HIV-1 infection. In this study, the neurorestorative potential of DAI and LQ was evaluated by monitoring their ability to prevent, and enhance recovery from, synaptodendritic injury produced *in vitro* by the HIV-1 protein, Tat 1-86B. The effects of DAI and LQ on restoration of the dendritic network were determined following withdrawal of Tat, and the role of estrogen receptors in the phytoestrogen-induced recovery process was determined through the use of tamoxifen. Collectively, these results determined the neuroprotective and neurorestorative potential of DAI and LQ treatment in HIV-1 Tat-induced synaptodendritic injury.

Methods

Primary neuronal cell culture

Primary cell cultures were prepared from embryonic 18 Sprague-Dawley rat fetuses as previously described (Aksenova et al., 2009; Bertrand et al., 2011). Timed-pregnant females were obtained from Harlan Laboratories, Inc., Indianapolis, IN, USA. In brief, hippocampal regions were dissected and incubated for 10 min in a solution of 2mg/mL trypsin in Hank's balanced salt solution (HBSS) buffered with 10mM HEPES (GIBCO Life Technologies, Grand Island, NY, USA). The tissue was then exposed to soybean trypsin inhibitor (1mg/mL in HBSS) and rinsed three times in HBSS. Cells were dissociated by trituration and distributed to poly-L-lysine coated glass bottom 35mm dishes (MatTek Corporation, Ashland, MA, USA) for cell viability measurements. Initial plating density in 96-well plates was approximately 160-180 cells/mm^2; in the 35 mm dishes the initial density was 120-140 cells/mm^2. A lower plating density was usedi n 35

mm dishes for clear identification of second order branching patterns. At the time of plating, culture dishes contained Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (GIBCO, Life Technologies) supplemented with 10% fetal bovine serum (Sigma Chemicals, St Louis, MO, USA). After a 24-h period, Dulbecco's modified Eagle's medium /Ham's nutrient mixture F-12 was replaced with an equal amount of Neurobasal (serum-free) medium, without phenol red, supplemented with 2% v/v B27, 2mM GlutaMAX supplement, and 0.5% w/v D-1 glucose (all ingredients from GIBCO). Cultures were maintained at 37°C in a 5% CO_2/95% room air-humidified incubator at all times. Serum-free culture medium was supplemented at weekly intervals. Cultures were used for experiments at the age of 21 days *in vitro* (DIV) and were >85-90% neuronal as determined by anti-MAP2/antGFAP/Hoescht fluorescent staining (Bertrand et al., 2011).

All animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of South Carolina (animal assurance number: A3049-01).

Experimental treatment of cell cultures.

The treatment of hippocampal cell cultures with HIV-1 Tat was carried out by the addition of 10µl freshly prepared stock solutions of recombinant Tat 1-86B (LAI/Bru strain of HIV-1 clade B. GenBank accension no. K02013) (Diatheva, Fano, Italy) to the serum-free cell culture growth medium (50nM final concentration). An equal volume of vehicle was added to control cultures. Prior work has shown that Tat 1-86B effects on the synaptodendritic arbor to be highly selective and dependent upon the presence of an intact cysteine domain, which is present in the HIV-1 Tat 1-86B (Bertrand et al., 2013).

Hippocampal cell cultures were treated with DAI (≥98.5% purity; Indofine Chemical Hillsborough, NJ, USA), LQ (≥98.5% purity; Indofine Chemical), and tamoxifen (TMX) (Tamoxifen citrate; Tocris Bioscience, Ellisville, MD, USA), DAI and

TMX were initially dissolved in dimethylsulfoxide and then diluted in phosphate-buffered saline (PBS). LQ was initially dissolved in methanol and then diluted in PBS. For the studies of acute injury and cell death, the cells were treated with DAI and LQ for 24 h prior to Tat treatment and assessed at either 24 h (F-actin puncta) or 48 h (Cell death). For recovery studies, DAI or LQ, (with or without TMX) were added to cultures 6 days after Tat treatment with assessments of F-actin puncta conducted 3 days later (i.e. 9 days after initial Tat treatment). TMX was used as initially described (Murphy & Segal, 1996) in studies of primary hippocampal cell cultures/spine morphology to modulate spine density. TMX was found to block the 17-β-estradiol-induced increase in spine density in the cultured hippocampal cells (Segal & Murphy, 2001), and not to increase neuronal process outgrowth or morphological complexity (O'Neill, Chen, & Brinton, 2004). For our hippocampal cell culture/morphological endpoints, the long-standing evidence in the literature supported the use of TMX.

Cell viability.

Hippocampal cell viability was assessed in primary hippocampal cell cultures prepared in 96-well culture plates (Costar) using the microplate reader-formatted variant of the fluorescent calcein AM/ethidium bromide cell labeling assay (Live/Dead kit; Invitrogen Life Technologies) as described previously (Aksenov et al., 2009; Aksenova et al., 2009; Adams et al., 2010; Adams et al., 2012). Initial cell viability measurements were carried out after 1, 24, 72, 96, and 144h incubation periods with Tat 1-86B (50nM). Subsequent cell viability measurements were carried out after 48-h incubation period with Tat 1-86B (50nM) which was preceded by a 24-h pre-treatment with DAI or LQ. Fluorescence was measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Data from treated cells were normalized to data from untreated

control cells in adjacent wells from the same plate and are presented as a percent of control. For cell viability tests, groups of 7-8 culture wells were analyzed. Two replicates for each experiment were performed using cell culture preparations from different animals.

Fluorescent labeling and immunocytochemistry.

The immunofluorescent labeling of primary hippocampal cell cultures was carried out in glass bottom cell culture dishes (MatTek Corporation). F-actin was visualized using a modified protocol for filamentous actin (F-actin) staining (Invitrogen Life Technologies). Briefly, treated and control cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and then treated for 20 min at 21°C with an F-actin specific probe, AlexaFluor 488 Phalloidin (1:40) (Invitrogen Life Technologies). Following F-actin labeling, cells were incubated with 10% normal horse serum (NHS) in PBS to block non-specific binding and co-labeled with rabbit polyclonal anti-MAP2 (1:1000) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) or anti-Tat rabbit polyclonal antibody (1:1000) (Diatheva) overnight. The secondary antibody for MAP-2 and Tat labeling was Alexa Red 594-conjugated goat anti-rabbit IgG (1:500, Invitrogen Life Technologies). Identification of cell nuclei was accomplished using direct Hoescht dye techniques (ImmunoChemistry Technologies LLC, Bloomington, MN, USA).

F-actin puncta.

Images of co-labeled F-actin/MAP-2 neurons were acquired using a high-resolution CCD camera attached to the Nikon Eclipse TE2000-E inverted fluorescent computer controlled microscope (20X objective, 1600 x 1200 pixel image size, 0.17 µm/px image resolution at 1X zoom; Nikon Instruments, Melville, NY USA). Between 3 and 5 Green (F-actin)/Red (MAP-2) immunolabeled/Blue (Hoescht) fluorescent images of individual

neurons with clearly defined dendritic arbors and normal nuclear morphology per culture well were randomly selected and analyzed using the NIS-Elements software package (Nikon Instruments). For each neuron selected, F-actin rich structures were identified in several (3-4) second order dendritic fragments (length range 25-75 µm) with continuous MAP-2 immunofluorescence. Fine folapodia, spine protrusions, and F-actin patches were considered F-actin rich structures; in contrast, growth cones (F-actin rich structures located at the most distal dendritic terminus) were excluded (Bertrand et al., 2013).

Trained independent observers manually counted F-actin puncta from identically treated cultures at different times, with a very high correlation (r^2=0.97), indicating that the green F-actin puncta were readily identified.

For computer-assisted methods, F-actin labeled puncta were identified by subtracting 20-50 au from the green channel to set the threshold for baseline staining of the dendritic shaft. Density was calculated by dividing total F-actin labeled puncta (N) by the length (L) of the MAP-2 labeled dendrites. For this study, puncta (size ≤1.5µm) of F-actin fluorescence with a peak intensity of at least 50% above the average intensity of staining in the dendritic shaft were included in each selected dendritic fragment.

Statistical analysis.

Statistical coparisons were made using ANOVA techniques with specific a priori contrasts and regressions, and were used to determine specific treatment effects. Pearson's product-moment correlation coefficient was calculated to verify inter-rater reliability and correlations between computer assisted profiling and manual counting. Comparisons and correlations were calculated using BMDP version 2009 (Statistical solutions, Saugus, MA, USA). Significant differences were set at $p \leq 0.05$.

GraphPad/Prism (V5.02; GraphPad Software, San Diego, CA, USA), was used for time course analyses.

Results

Tat 1-86B synaptodendritic injury and cell death time course.
After treatment with 50 nM Tat 1-86B cell viability was monitored at 1, 24, 48, 72, 96, and 144 h (Fig. 3.1A). Tat 1-86B failed to induce significant cell death through 24h. Tat 1-86B induced significant cell death at 48 h ($p< 0.001$). Cell death reached a plateau at 72 h with no additional changes at 96 or 144 h.

Cell cultures were co-stained for F-actin and MAP-2 at 1, 24, 72, and 144 h after addition of Tat (50nM). Tat exposure resulted in degradation of pyramidal cell dendrites, indicated by loss of F-actin staining (Fig. 3.1B). In addition to simplification of the cellular network, the presence of fibril bundling and fragmentation suggested microtubule dysfunction. Tat 1-86B mediated F-actin network degradation occurred earlier than Tat-mediated cell death, with degradation observed at 24 h.

Figure 3.1C illustrates the association of Tat 1-86B (red) with the neuronal network (green) at 1 and 24 h in primary hippocampal cell cultures. Subsequent acute experiments were performed at 24 h because of the visible change in F-actin rich structures, lack of overt cell death, and persistence of Tat 1-86B within the neuronal network.

Computer assisted identification of F-actin puncta.
Computer-assisted intensity profiling was used to determine the number of F-actin rich structures within selected dendritic segments. Figure 3.2A shows representative selections of Tat 1086B treated and vehicle treated controls. The computer-assisted

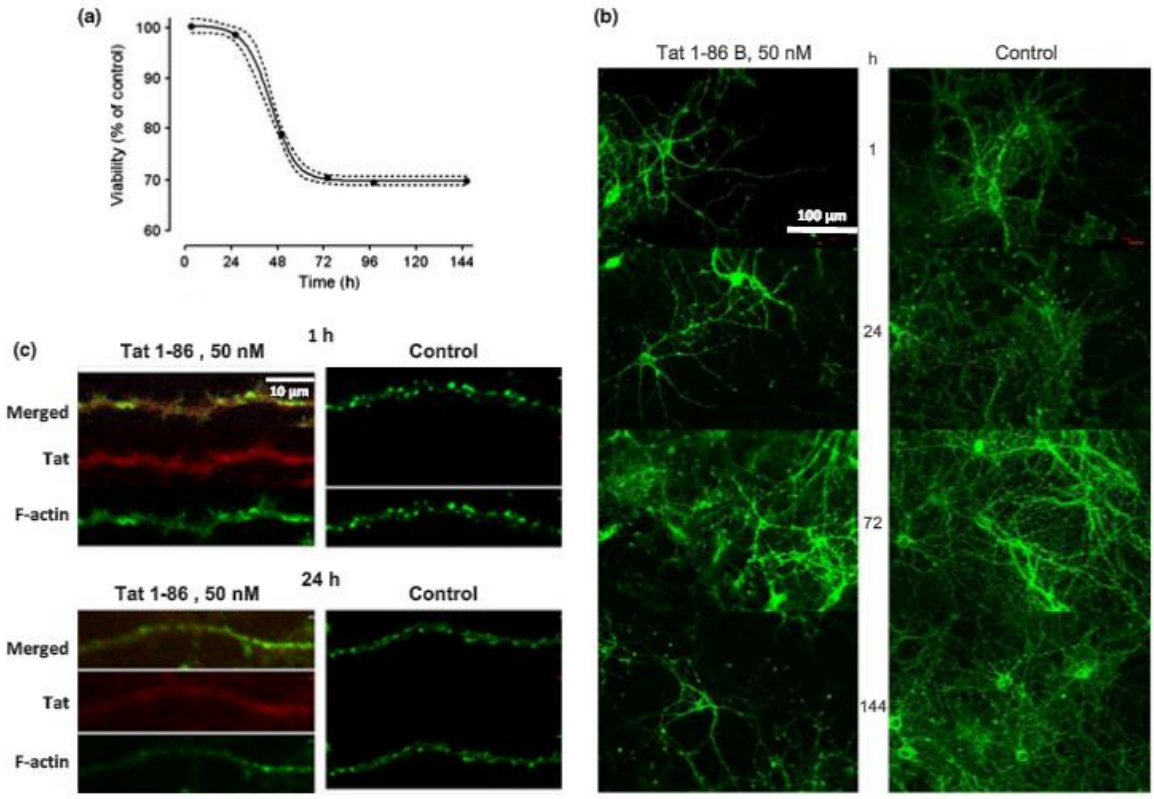

Figure 3.1 Time-course of Tat-induced cell death and synaptodendritic alterations. **A.** The viability of primary cultures was assessed at 1, 24,48, 72, 96, and 144 h after treatment with Tat. There was no significant cell death through 24 h; however, by 48 h there was a significant decrease in cell viability ($p \leq 0.001$). By 72 h, cell death reached a plateau and remained stable through 144 h. Mean ± 95% confidence interval (CI). **B.** F-actin/MAP-2 staining of cell cultures at 1, 24,72, and 144h after Tat 1-86B exposure. Tat 1-86B treatment produced a simplification of the fine network, as well as bundling and fragmentation of neurites at 24 h, which preceded Tat-mediated cell death. Tat-induced alterations in the neuronal network persisted through 144 h. **C.** Representative images (20X) of second branch order neuritic fragments co-stained with Alexa 488 phalloidin and rabbit polyclonal anti-Tat primary/Alexa Red 594. Tat was associated with the F-actin labeled network at 1h and 24h post-treatment.

profiles of MAP-2 immunofluorescence (25-75µm length; intensity range 37-110 arbitrary units, au) in cultures were continuous, indicating the absence of fragmentation of MAP-2 labeled dendrites, regardless of treatment group. The peak F-actin fluorescence (puncta) varied from 60-200 au within the dendritic segments.

As shown in Fig. 3.2b, F-actin rich puncta per 10µm of F-actin/MAP-2 labeled dendrite, was significantly (non-overlapping distributions) decreased in neurons exposed

to 50 nM Tat for 24h using both computer-assisted and manual counting methods. There was a very high correlation between the two methods (r^2=0.97).

Phytoestrogens are not neurotoxic at 1.0µM doses.

An overall significant effect of phytoestrogens in protecting against Tat-induced cell death was suggested (F(1,71)=54.4, $p\leq0.0$01), with a most prominent linear effect of dose (F(1,71)=166.4, $p\leq0.0$01) and a less prominent quadratic effect of dose (F(1,71) =7.3, $p\leq0.0$01).

To determine the ability of DAI to provide protection against neuronal death, cells were pre-treated with DAI for 24 h and then incubated with Tat 1-86B for 48h. There was an overall significant main effect of DAI treatment on viability F(4,36)= 40.2, $p\leq0.0$01, as pre-treatment with DAI protected against Tat-induced toxicity (Fig. 3.3A). Planned comparisons indicated that cell viability following treatment with 1.0µM DAI alone was not significantly different from untreated control cultures (F<1.0), suggesting that DAI alone did not produce neurotoxicity at the highest dose. A quadratic dose-dependent effect of DAI was found (F(1,36)=32.6, $p\leq0.0$01), with complete neuroprotection achieved, as pre-treatment with DAI prevented Tat 1-86B induced cell death in both the 0.2 and 1.0µM DAI treatment groups (Fs(1,36) >78.0, ps <0.001).

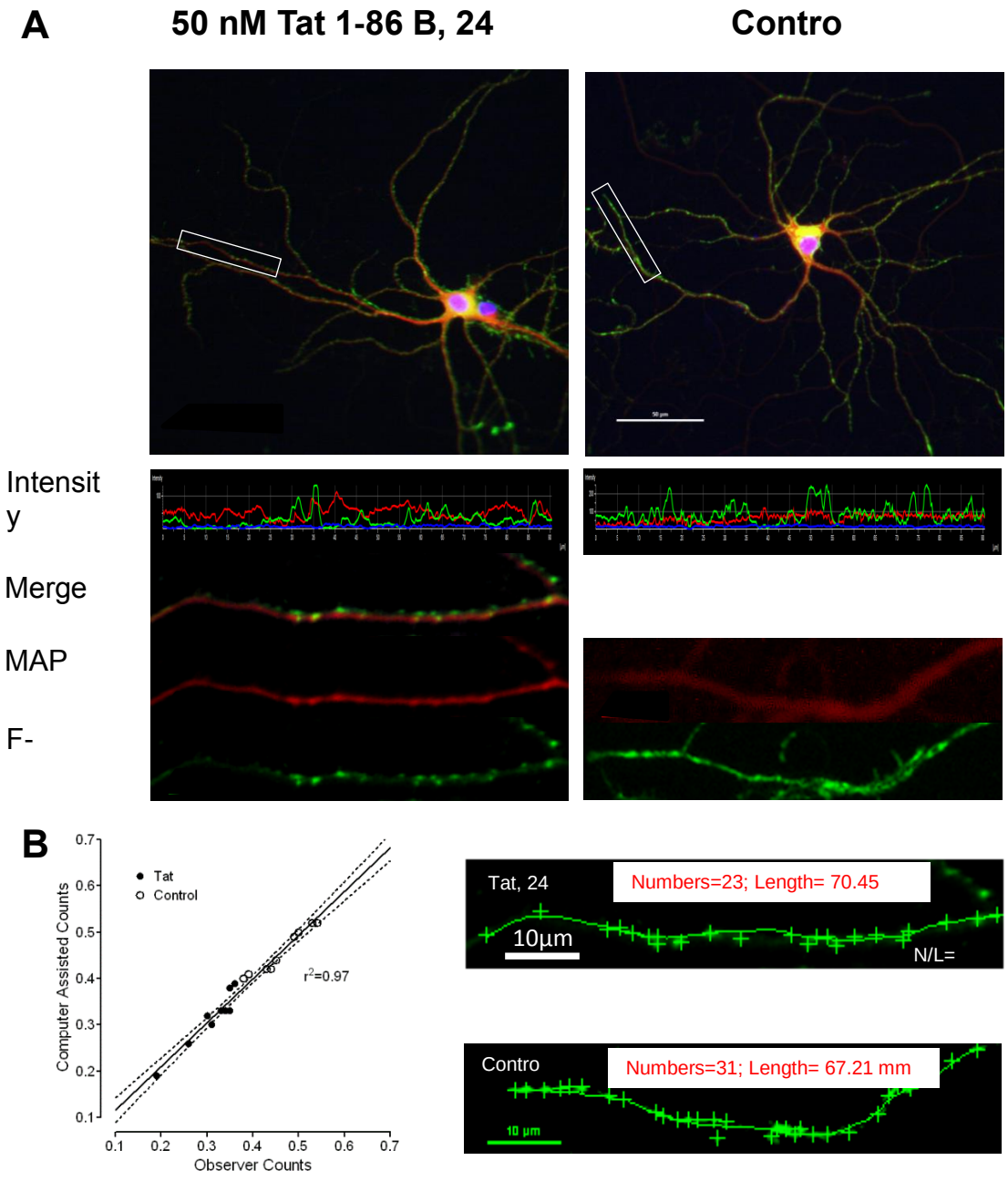

Figure 3.2 Computer-assisted identification of F-actin puncta after Tat treatment. **A.** Segments of second order dendritic branches (shown as boxes) were selected from 20X images of F-actin/MAP2/Hoescht-stained Tat-treated and non-treated control neurons. The computer-assisted intensity profile of F-actin fluorescence (green) showed numerous peaks corresponding to F-actin rich structures and valleys corresponding to low baseline staining of dendritic shafts. The computer-based intensity profile of MAP2-specific immunofluorescence (red) showed uniform labeling of all dendritic segments selected for counting of the F-actin puncta (N; green+) was divided over the length of the neurite (L;

green line) to provide the mean number of F-actin labeled puncta per 10µm of neuronal dendrite ±SEM. **B.** F-actin puncta were significantly reduced in neurons exposed to Tat for 24 h using both computer-assisted intensity profiling and manual counting methods. F-actin puncta are readily distinguished and there was a very high correlation between the two methods (r^2=0.97).

To determine the ability of LQ to provide protection against cell death, cells were pretreated with LQ and then incubated with Tat 1-86B for 48h. There was an overall significant main effect of LQ treatment on viability $F(4,35) = 25.7$, $p \leq 0.001$, as pre-treatment with LQ protected against Tat-induced toxicity (Fig. 3.3B). There was no significant difference between LQ-treated cultures and controls, indicating that LQ was not neurotoxic ($F < 1.0$). A linear dose-dependent effect of LQ was found $F(1,35)= 77.6$, $p < 0.001$, with significant, although not complete, neuroprotection. Specifically, planned comparisons indicated that pre-treatment with LQ provided significant prevention of Tat-mediated cell death in both the 0.1 and 1.0µM LQ dose groups ($Fs(1,35) > 7.2$, $ps<0.01$), but that both LQ dose groups displayed significantly decreased cell viability relative to controls ($Fs(1,35) > 7.6$, $ps< 0.009$). Based on the lack of neurotoxicity of 1.0 DAI and 1.0 LQ (i.e. phytoestrogen treatments not different from untreated controls), the 1.0µM doses were used in subsequent studies of dendritic recovery.

Phytoestrogen pre-treatment provides protection against acute HIV-1 Tat mediated synaptodenritic injury.

An overall significant effect of phytoestrogens protecting against acute Tat-induced synaptodendritic injury was suggested by the significant phytoestrogen treatment effect ($F(1,58)=8.9$, $p <0.004$) and the significant interaction between Tat and phytoestrogen treatments ($F(1,58) = 4.9$, $p \leq 0.03$), without any significant difference between the two phytoestrogens (DAI vs LQ) in their neuroprotection ($Fs < 1.0$).

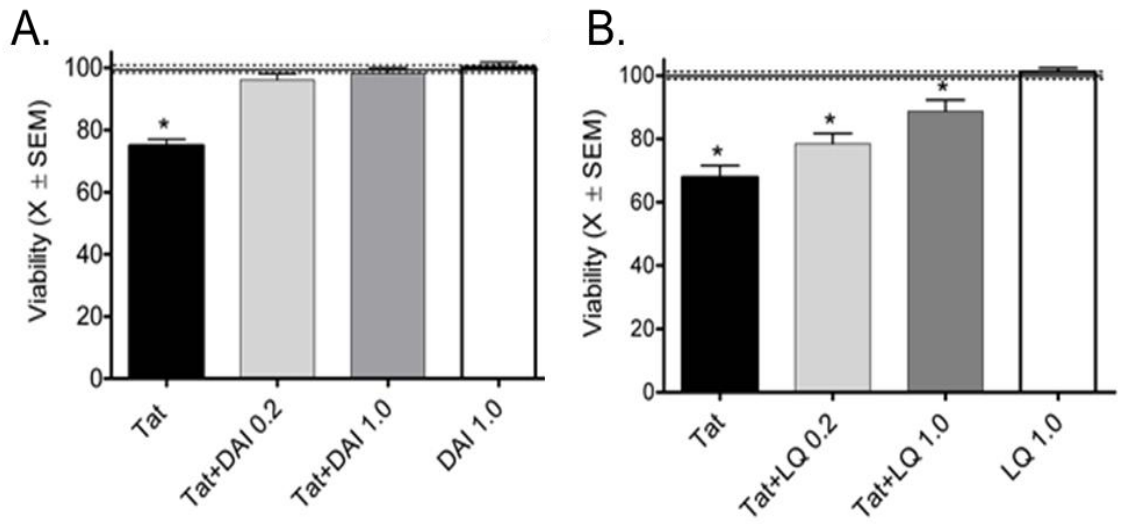

Figure 3.3 Pre-treatment with phytoestrogens provides dose-dependent protection against neuronal cell death. **A.** Cell viability after daidzein (DAI) 24 h pre-treatment and a 48 h incubation with 50nM Tat 1-86B. Treatment with 1 µM DAI alone had no significant effect on cell viability relative to control cultures (F<1.0). DAI pre-treatment provided complete neuroprotection in both the 0.2 µM and 1 µM treatment groups (ps<0.001). Results are presented as mean % of control ± SEM. *Indicates significant protection of Tat-induced neurotoxicity. **B.** Cell viability after 24 h liquiritigenin (LQ) pre-treatment and a 48h incubation with 50nM Tat 1-86B. There was no significant difference between LQ-treated cultures and controls, indicating 1 µM LQ was not neurotoxic. A linear dose-dependent effect of LQ was found (p≤0.001) with significant, although not complete, neuroprotection. Results are presented as mean % of control ± SEM. * Indicates significant protection of Tat induced neurotoxicity.

Daidzein. Fine network integrity was assessed by counting F-actin puncta after pre-treatment with DAI (Fig. 3.4A) and then 24h of incubation with Tat 1-86B (Fig. 3.4B). Planned contrasts indicated that DAI was not significantly different from controls (F < 1.0), suggesting that DAI was not significantly different from controls (F < 1.0), suggesting that DAI was neither toxic nor stimulatory. However, Tat 1-86B produced a significant loss of F-actin puncta (F(1,31) = 10.2, <0.003), and DAI provided significant protection against the synaptodendritic damage/puncta loss caused by Tat (F(1,35)= 4.7, p < 0.039).

Representative images (Fig. 3.4c-e) show that relative to control cultures (Fig. 3.4c), Tat treatment induced a simplification of the network (Fig. 3.4d). Moreover, there was no significant difference between DAI+Tat treated cultures and controls (Fig. 3.4e), indicating that DAI pre-treatment protected against F-actin puncta loss induced by Tat 1-86B.

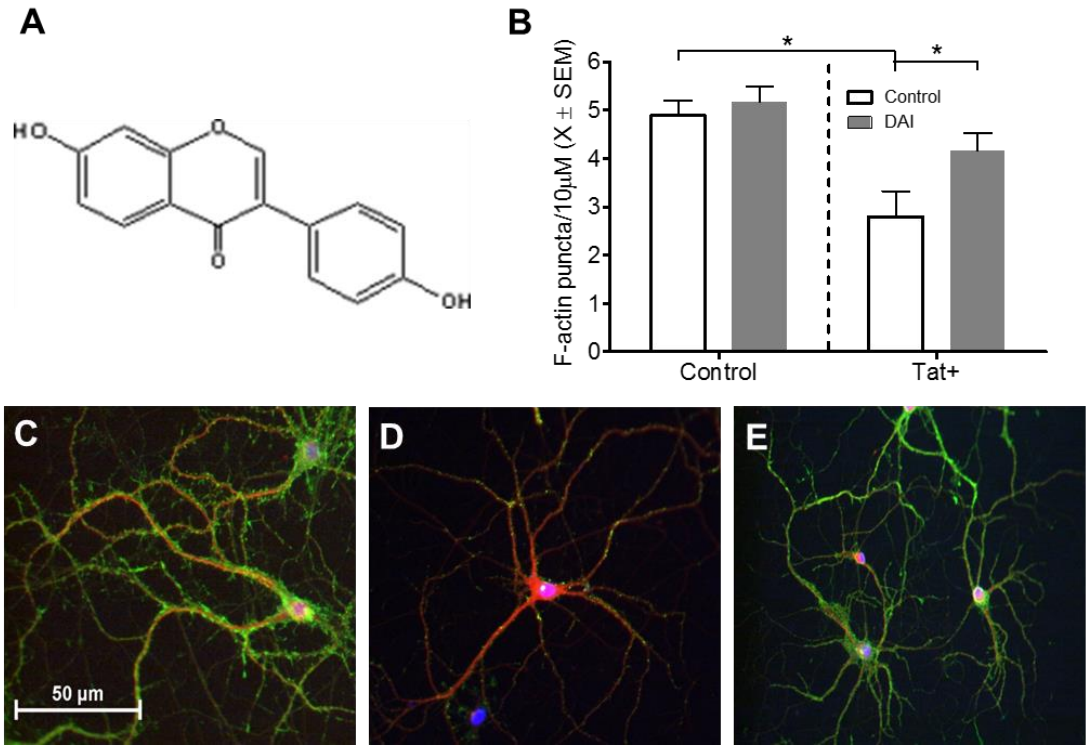

Figure 3.4 Pre-treatment with DAI protects against Tat-induced loss of F-actin puncta. **A.** Chemical structure of daidzein (7,4'-Dihydroxyisoflavone). **B.** F-actin puncta following pre-treatment (24h) with 50nM Tat 1-86B. DAI was neither toxic nor stimulatory to production of F-actin puncta; however Tat treatment produced a significant loss of F-actin puncta ($p \leq 0.003$), and DAI provided significant protection against the puncta loss caused by Tat. Results are presented as mean number of F-actin labeled puncta per 10µm of neuronal dendrite ± SEM. *Indicates 1 µM DAI pre-treatment provided significant protection from damage induced by Tat 1-86B (50nM; 24 hours) when compared with cultures treated with Tat 1-86B alone. **C-E.** Neurons from **C**. vehicle-treated control cultures, **D.** Tat 1-86B-treated (50nM; 24h) cultures, and **E.** pre-treated DAI (1µM; 24 h) + incubated with Tat 1-86B (50nM; 24 h) cultures displaying typical F-actin (green), MAP-2 (red) and Hoescht (blue) staining for each treatment group. The control image shows robust F-actin presence, complex branching patterns, and an extensive fine network. Tat 1-86B treatment induced a simplification of the network. In contrast, in the DAI pre-treated culture, Tat 1-86B failed to cause network

simplification, suggesting DAI pre-treatment protected against Tat-induced synaptodendritic alterations.

Liquiritigenin.

To determine the ability of LQ to provide synaptodendritic protection, cultures were pre-treated with LQ to provide synaptodendritic protection, cultures were pre-treated with LQ (Fig. 3.5A) and then incubated with Tat 1-86B (Fig. 3.5B). Planned comparisons indicated that pre-treatment with LQ did not alter puncta density from controls (F<1.0), suggesting that LQ was neither toxic nor stimulatory. However, Tat 1-86B again produced a significant loss of F-actin puncta (F(1,27)=13.8, $p \leq 0.001$), and LQ provided significant protection against the synaptodendritic damage/puncta loss caused by Tat (F(1,27)=6.7, $p \leq 0.016$).

Representative images are shown in Fig. 3.5C-E. Relative to controls (Fig. 3.5C), Tat treatment produced significant damage to the network (Fig. 3.5D). The difference between LQ+Tat-treated cultures and controls was not significant (Fig. 3.5E), indicating that the pre-treatment with LQ protects against Tat 1-86B-induced loss of F-actin rich structures.

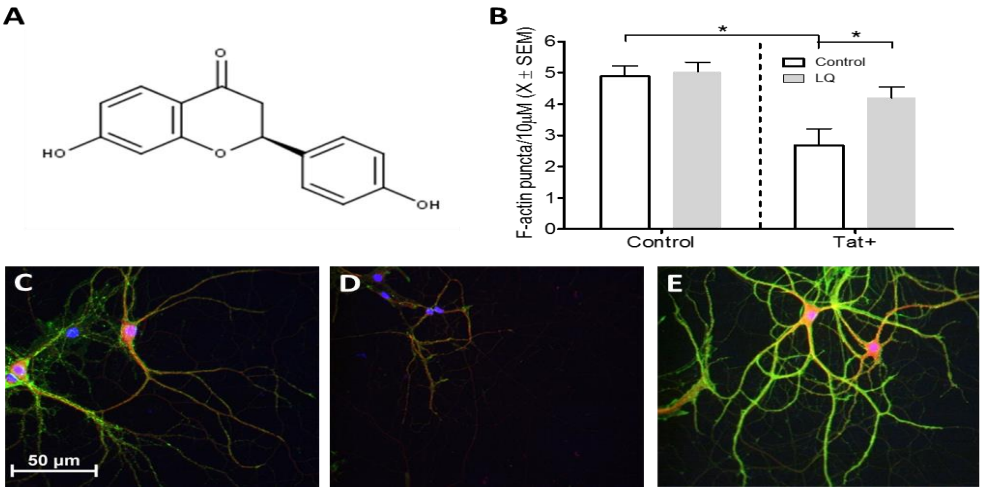

Figure 3.5 Pre-treatment with liquiritigenin (LQ) provides protection against Tat-induced loss of F-actin puncta. **A.** Chemical structure of liquiritigenin (4',7-Dihydroxyflavanone). **B.** Quantification of F-actin puncta with a pre-treatment (24 h) of 1μM LQ and 50 nM

Tat 1086B (24h). LQ did not alter F-actin puncta density from control (F<1.0), suggesting that LQ was neither toxic nor stimulatory. Tat treatment produced a significant loss of F-actin puncta density from control (*F*<1.0), suggesting that LQ was neither toxic nor stimulatory. Tat treatment produced a significant loss of F-actin puncta ($p \leq 0.001$) and LQ provided significant protection against the puncta loss cuased by Tat ($p \leq 0.016$). Results are presented as mean number of F-actin labeled puncta per 10 µm of neuronal dendrite ± SEM. *Indicates a significant loss of F-actin puncta after Tat 1-86B treatment when compared with vehicle-treated controls ($p \leq 0.01$). *Indicates 1µM LQ pre-treatment provided significant protection from F-actin puncta loss induced by 1-86B (50nM; 24 h) when compared with cultures treated with Tat 1-86B alone. **C-E.** Neurons from **C.** Vehicle-treated control cultures, **D.** Tat 1-86B-treated (50nM; 24 h) cultures, and **E.** pre-treated LQ (1µM; 24 h) + incubated with Tat 1-86B (50nM; 24 h) cultures, displaying typical F-actin (green), MAP-2 (red) and Hoescht (blue) staining for each treatment group. The control image shows robust F-actin presence, complex branching patterns, and an extensive fine network. Tat 1-86B treatment induced a simplification of the dendritic network. In contrast, in the LQ pre-treated culture, Tat 1-86B failed to cause network simplification, indicating that LQ pre-treatment protected against Tat-induced synaptodendritic alterations.

Neurorestoration of prior HIV-1 Tat-mediated synaptodendritic injury via Daidzein and Liquiritigenin.

After the cytotoxic effect of Tat was fully developed (6 days), the Tat-containing medium was replaced with fresh medium (i.e. without Tat). Three days after medium replacement, a significant decrease ($F(1,64)=32.2$, $p \leq 0.001$) in the F-actin puncta remained in cultures with prior Tat exposure, relative to controls (Fig. 3.6). The cell cultures treated with DAI, LQ, or TMX were not significantly different from control (*Fs*<1.0), suggesting a lack of toxicity from these compounds. However, when either 1.0 µM LQ or DAI were included in the replaced medium, a significant increase in F-actin puncta was found ($F(1,64)=10.6$, $p \leq 0.002$). To determine if the DAI or LQ neurorestoration was dependent on ER-signaling, a similar experiment was performed in the presence of tamoxifen (TMX). Inclusion of TMX with either DAI or LQ in the cultures with prior Tat exposure blocked the restoration of F-actin puncta by the phytoestrogens ($F(1,64)=8.0$, $p \leq 0.006$).

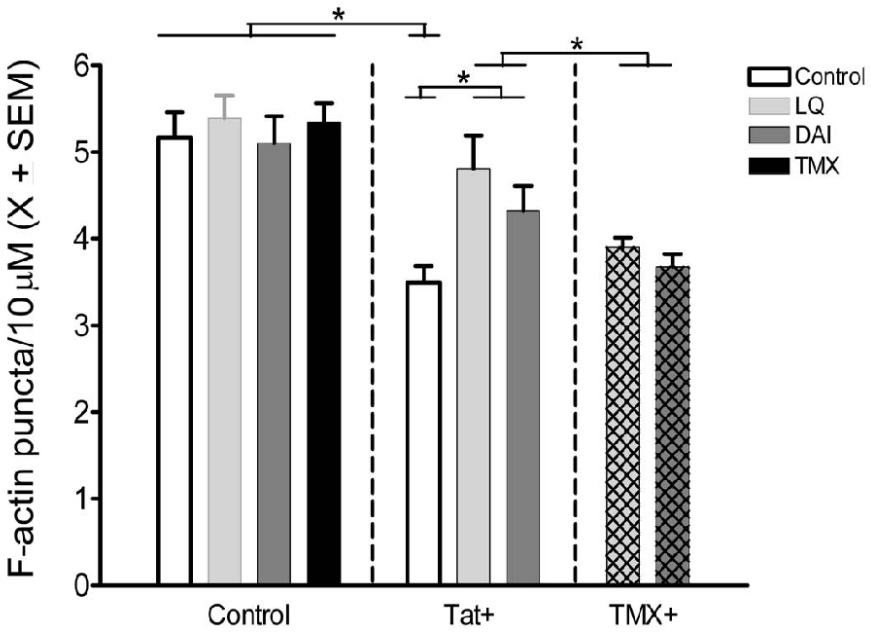

Figure 3.6 Phytoestrogens enhance the recovery of F-actin puncta from HIV-1 Tat-induced synaptodendritic injury in an estrogen receptor-dependent mechanism. The neurorecovery experiment was initiated by medium replacement after 6 days (i.e. after the initial cytotoxic effects of Tat). Three days after medium replacement (9days after initial Tat treatment) a significant loss of F-actin puncta remained ($p \leq 0.001$) in Tat-treated cultures. The cull cultures treated on day 6 with either daidzein (DAI), liquiritigenin (LQ) or tamoxifen (TMX were not significantly different from controls. When either DAI or LQ were added on day 6 to the cultures initially treated with Tat, a significant increase in F-actin puncta was detected ($p \leq 0.002$). This enhancement by DAI or LQ was blocked by the estrogen receptor antagonist TMX (100nM), suggesting involvement of estrogen receptors in mediating the recovery of F-actin puncta. Results are presented as mean number of F-actin labeled puncta per 10 µm of neuronal dendrite ± SEM. *Indicates significant differences ($p \leq 0.05$) between indicated groups.

Discussion

The major findings of this study are (i) acute pre-treatment with either LQ or DAI, two plant-derived phytoestrogenic compounds, prevents the loss of F-actin puncta induced by HIV-1 Tat. Synaptodendritic damage was quantified by the significant loss of F-actin puncta after a short exposure to Tat. And Tat was found to be acutely associated with the dendritic arbor. Moreover, (ii) these phytoestrogens were able to promote

restoration of F-actin dendritic puncta and dendrites following damage from HIV-1 Tat in hippocampal cells. This recovery occurred via an estrogen receptor-mediated mechanism, as restoration of F-actin puncta was blocked by tamoxifen. These results suggest that DAI, LQ, and compounds of similar chemical structures, could be beneficial in HIV-1 therapeutics because of their ability to either prevent, and/or enhance recovery, from synaptodendritic injury.

The brain serves as a reservoir for HIV-1, as cART effectively suppresses viral replication in the periphery, but does not eradicate the virus from the brain (Heaton et al., 2010). Synaptodendritic injury has been found in latent HIV-1 infection, in which active viral replication is not present in the brain, despite the presence of proviral DNA. Latent HIV-1 infection in humans produces dendritic loss (decreased MAP2 staining) and synaptic loss (decreased synaptophysin staining) in the frontal cortex without viral replication (Desplats et al., 2013). HIV-1 proviral DNA may produce early proteins, such as Tat, without productive viral infection (Wu, 2004). In this study, the co-localization of Tat within the F-actin rich dendritic network and the concomitant loss of puncta suggests a mechanistic interaction between Tat and synaptodendritic injury, however it is currently unknown whether the synaptodendritic loss in human latent HIV-1 infections is caused by Tat expression *per se*. Nevertheless, our *in vitro* model of HIV-1 Tat exposure demonstrates dendritic loss (decreased MAP2 staining) and synaptic loss (decreased F-actin puncta), similar to that observed in post-mortem tissue from human brain with latent HIV-1 infection.

F-actin puncta encompass a variety of neuronal structures, the most well-understood being dendritic spines. Dendritic spines are F-actin rich protrusions from the

dendritic arbor, are the sites of post-synaptic excitatory synapses, and change shape rapidly in response to extracellular signaling (Kaech et al., 1997; Calabrese, Wilson, & Halpain, 2006; Hotulainen et al., 2009; Dent et al., 2011). Non-spiny synapses, typically GABA-ergic (Craig et al., 1994), have also been found to contain a significant concentration of F-actin and appear as patches or 'hot spots' on the dendritic arbor (Halpain et al., 1998; Lau et al., 1999). We found that intensity profiling, a semi-automatic computer-based method, and manual counting produced similar puncta densities *in vitro*. As a result of the varying morphology spines, the ability of F-actin staining to detect non-spiny synapses, and the early filamentous stages of spinogenesis, the quantification of all F-actin rich structures, or puncta, provides a measurement of overall synaptic integrity, healthy of the dendritic network, and potential to recover from Tat-induced injury.

Although F-actin is known to play a role in spinogenesis and synaptic plasticity, the staining of F-actin is a relatively new technique used to image dendritic spines and monitor synaptic integrity (Matus, Ackermann, Pehling, Byers, & Fujiwara, 1982; Allison, Gelfand, Spector, & Craig, 1998; Sekino et al., 2007). In comparison, the Golgi method has been used for well over a century to identify changes in spine morphology and density, and is still in use. The Golgi method has been favored over the years because of the particular ability of Golgi to produce images with little to no background and complete neuronal filling. However, Golgi randomly and unpredictably stains neurons and has been found to significantly underestimate spine number and, moreover, fails to identify non-spiny synaptic structures and patch morphology (Mancuso, Chen, Li, Xue, & Wong, 2012). Non-spiny inhibitory synapses, which can be detected as patches by F-

actin staining, but are not detected by the Golgi method, are important for normal communication between neurons (Heller et al., 2012).

In this sudy, we used F-actin to assess the synaptodendritic injury induced by HIV-1 Tat, whereas other studies have used Golgi (Sa et al., 2004; Fitting et al., 2010), green fluorescent protein coupled to post-synaptic density 95 (GFP-PSD95) labeling (Kim et al., 2008a; Shin, Kim, & Thayer, 2012b), or MAP-2 staining (Maragos et al., 2003) to evaluate the damage to the dendritic arbor. Golgi detects changes in spine morphology, but not changes in non-spy synapses or thin filopodia. GFP-PSD95 staining can detect only post-synaptic excitatory structures, as GFP-PSD95 staining can detect only post-synaptic excitatory structures, as PSD-95 has not been found in non-spiny inhibitory synapses (Heller et al., 2012) and is not found in pre-synaptic structures. Although pre- and post-synaptic terminals generally correspond to one another (Craig et al., 1994), PSD-95 can still be located after F-actin has disappeared from the spine (Halpain et al., 1998), suggesting that F-actin may be a more sensitive marker. MAP-2 staining can detect changes in overall structure of the neuronal arbor, but cannot reliably detect subtle changes in spine or patch morphology as microtubules have been observed to enter dendritic spines selectively and microtubule entry is activity dependent (Dent et al., 2011). Although the aforementioned techniques vary, and the studies determined the effects of HIV-1 Tat both *in vivo* (Maragos et al., 2003; Fitting et al., 2010) and *in vitro* (Shin et al., 2012b; Kim et al., 2008a), collectively these studies indicate that HIV-1 Tat produces dendritic damage. Therefore, identifying mechanisms or compounds that either prevent or reverse synaptodendritic damage inflicted by HIV-1 Tat may be useful approaches for providing neuroprotection during HIV-1 infection.

As a potential therapeutic approach for recovery of synaptodendritic damage, estrogen has long been known to rapidly increase dendritic spine density *in vivo* (Gould, Woolley, Frankfurt, & McEwen, 1990; Woolley & McEwen, 1992), specifically through regulation of the actin cytoskeleton in spines (Sanchez et al., 2009). Phytoestrogens are non-steroidal, diphenolic structures found in plants that have similar chemical and structural properties to 17-β-estradiol (Glazier & Bowman, 2001; Lephart et al., 2005). We found that phytoestrogens promote recovery from the synaptodendritic injury is correlated with the symptoms of HAND (Ellis et al., 2007; Desplats et al., 2013), the extent to which synaptic restoration is possible remains unknown. Also unknown is whether there might be a critical therapeutic window for promoting effective recovery wherein neurorestoration remains possible. Unfortunately, studies of therapeutic pathways for enhancing dendritic recovery are few (Kim et al., 2008a; Shin et al., 2012b); however, estrogen has been shown to promote spine formation via modulation of F-actin in spines (Kramar et al., 2009; Sanchez et al., 2009). The current data suggest that phytoestrogens, possibly acting via F-actin, may provide a novel intervention for promoting neurorestoration. The activation of estrogen receptors has been shown to play a key role in modulation of dendritic spine dynamics (Liu et al., 2008; Kramar et al., 2009; Sanchez et al., 2009; Srivastava, Woolfrey, Liu, Brandon, & Penzes, 2010; Phan, Lancaster, Armstrong, MacLusky, & Choleris, 2011), suggesting the estrogen receptor may be a useful target in ameliorating synaptodendritic injury, such as that seen in HAND.

Previous studies have shown that LQ is a highly specific ERβ agonist, with a 75-fold binding preference to ERβ over ERα (Mersereau et al., 2008) and only activates ERβ

(Kupfer et al., 2008; Paruthiyil et al., 2009). DAI preferentially binds to ERβ (Zhao, Mao, & Brinton, 2009). The selectivity of LQ for ERβ suggests that LQ acts through an ERβ dependent mechanism to prevent synaptodendritic damage induced by HIV-1 Tat; however, more experimentation is needed to determine the receptor-dependent mechanism of LQ.

Our results illustrate that HIV-1 Tat causes an early reduction in F-actin positive puncta. Interventions aimed at promoting synaptodendritic integrity following HIV-1 infection of the nervous system would therefore appear to be an effective approach for preventing HAND. Moreover, our results indicate that damage by HIV-1 Tat may be repaired by the phytoestrogens, liquiritigenin and daidzein, and estrogen receptor actions mediate this neurorestoration. Although it is presently unknown if such reversals can improve neurocognitive outcomes, phytoestrogenic compounds, like DAI and LQ, may prevent cumulative injury to the dendritic network, and ultimately, aid recovery from HIV-associated neurocognitive disorders

CHAPTER 4

HIV-1 TAT AND COCAINE MEDIATED SYNAPTODENDRITIC INJURY IS PREVENTED BY S AND R EQUOL[3]

[3] SJ Bertrand, C Hu, MV Aksenova, CF Mactutus, RM Booze. To be submitted to *Frontiers in Microbiology*.

Introduction

The use of illicit drugs, like cocaine, significantly increases the risk of HIV-1 transmission, and continued drug use may compound neurological complications. Individuals that use cocaine during HIV-1 infection progress from HIV-1 to AIDS more quickly (Fiala et al., 1998; Baum et al., 2009), are more likely to develop neurocognitive disorders (Nath et al., 2001; Levine et al., 2006; Carey et al., 2006; Rippeth et al., 2004), and display an accelerated progression of HAND (Nath et al., 2001). Neurocognitive dysfunction and drug use are two of the most common factors for medication non-compliance (Panos et al., 2014), thus these individuals have a higher risk for disease progression, increased mortality rates, and a lower quality of life (Baum et al., 2009; Cook et al., 2007; Doshi et al., 2012; Qian et al., 2014; Nath et al., 2001; Nath, 2010; Wisniewski et al., 2005).

Effective combined anti-retroviral therapy (cART) results in a lack of active viral replication. However, infected astrocytes and microglia may continue to release neurotoxic proteins, like Tat and gp120, into the extracellular space (Desplats et al., 2013). HIV-1 proteins interact with neurons to produce dysfunction and damage (Desplats et al., 2013; Everall et al., 1999; Masliah et al., 1997; Moore et al., 2006). Neuronal damage is predictive of pre-mortem cognitive status than neuronal cell death in HAND (Desplats et al., 2013; Everall et al., 1999; Adle-Biassette et al., 1999; Kaul et al., 2001). *In vitro*, HIV-1 Tat and gp120 produce synaptodendritic damage prior to overt cell death (Kim et al., 2008a; Bertrand, Mactutus, Aksenova, Espensen-Sturges, & Booze, 2014; Bertrand et al., 2013) and synaptodendritic injury may be a reversible process (Bertrand et al., 2014; Kim et al., 2008a).The combination of HIV-1 neurotoxic proteins,

like Tat, and cocaine have been shown to enhance neuronal cell death *in vitro* (Turchan et al., 2001;Kendall et al., 2005), however it is unclear how HIV-1 proteins and cocaine may interact at the synaptic level to produce damage early in disease.

Pre- and post-synaptic structures are rich in actin, a dynamic cytoskeletal protein (Zhang & Benson, 2001; Hotulainen et al., 2009; Johnson & Ouimet, 2006; Sekino et al., 2007). Subsequent to cellular signaling, globular actin (G-actin) is polymerized into filamentous actin (F-actin), resulting in a stable synaptic structure (Hotulainen et al., 2009; Johnson & Ouimet, 2006; Sekino et al., 2007). Stabilized synaptic structures are rich in F-actin and appear as spots or puncta; presently, we define F-actin puncta as protruding dendritic spines, different stages of spine development (patch, filopodia, protruding spines) (Hotulainen et al., 2009; Johnson & Ouimet, 2006; Matus et al., 1982; Sekino et al., 2007), non-spiny or inhibitory synapses (Heller et al., 2012), and pre-synaptic structures (Sankaranarayanan, Atluri, & Ryan, 2003; Halpain, 2003). The quantification of F-actin puncta allows an overall evaluation of synaptic integrity *in vitro*. Since F-actin puncta encompass a variety of post-synaptic structures and play a role in pre-synaptic vesicle stability (Sankaranarayanan et al., 2003; Halpain, 2003) a change in overall puncta number suggests altered synaptic communication.

HIV-1 Tat produces a reduction in dendritic spines *in vitro* (Kim et al., 2008a) and *in vivo* (Fitting et al., 2010), and produces a reduction in F-actin rich puncta prior to cell death *in vitro* (Bertrand et al., 2014; Bertrand et al., 2013). Indeed, HIV-1 Tat increases actin depolymerization in endothelial cells (Wu et al., 2004), and disrupts microtubules to induce apoptosis (Chen, Wang, Zhou, & Zhou, 2002). HIV-1 Tat also alters NMDA receptor calcium currents via interactions with the actin cytoskeleton

(Krogh, Lyddon, & Thayer, 2015). Although it is clear that HIV-1 Tat interacts with the actin cytoskeleton, it is unclear if this is a concentration dependent process.

Cocaine has been shown to cause an increase in the expression of F-actin (Kalivas, 2009), and increase dendritic spine density (Martin et al., 2011). Indeed, inhibiting the polymerization of G-actin into F-actin prevents morphological changes found in the nucleus accumbens of rats acutely treated with cocaine (Toda, Shen, & Kalivas, 2010). Although the combination of HIV-1 proteins and cocaine results in increased levels of neuronal cell death (Kendall et al., 2005; Turchan et al., 2001), the contradictory effects of HIV-1 Tat and cocaine on F-actin make the combined effects of HIV-1 Tat and cocaine difficult to predict.

Estrogen prevents neuronal apoptosis and degeneration in several neurodegenerative diseases (Chakrabarti et al., 2014), modulates concentrations of F-actin in dendritic spines (Sanchez et al., 2009; Kramar et al., 2009), and prevents apoptosis in HIV-1 Tat treated cultures (Adams et al., 2010). Furthermore, estrogen prevents enhanced levels of neuronal cell death mediated by HIV-1 proteins (Tat, gp120) and cocaine *in vitro* (Kendall et al., 2005; Turchan et al., 2001). However, estrogen does not selectively bind and interacts with various receptor subtypes including alpha (ERα), beta (ERβ), and G-protein coupled receptor-30 (GPR-30). ERα and ERβ are classic steroid receptors; they are located intracellularly where they translocate to the nucleus following activation (O'Lone, Frith, Karlsson, & Hansen, 2004). GPR-30 is a membrane receptor, responsible for some of the more immediate effects of estrogen (Raz, Khan, Mahesh, Vadlamudi, & Brann, 2008), although these effects are not well understood. There are high concentrations of ERα in reproductive tissue which has associated ERα

activation with the proliferative effects of estrogen. Meanwhile, ERβ is highly expressed in the brain (Shughrue, Lane, & Merchenthaler, 1997b), and has been found in extranuclear sites (Yang et al., 2004; Sheldahl, Shapiro, Bryant, Koerner, & Dorsa, 2008; Herrick, Waters, Drake, McEwen, & Milner, 2006) making it a more likely target for neuroprotection. Specific activation of ERβ, or potentially GPR-30, may bypass the unwanted side effects produced by ERα.

Phytoestrogens are a class of naturally occurring compounds that are structurally and functionally similar to mammalian estrogen (Glazier & Bowman, 2001; Lephart et al., 2005). Soy derived phytoestrogens daidzein and genestein are neuroprotective (Chen et al., 2008; Mao et al., 2007), have been used to treat various types of cancer, cardiovascular diseases, osteoporosis (Jackson et al., 2011), prevent HIV-1 Tat induced neuronal apoptosis (Adams et al., 2012), and prevent and reverse synaptodendritic injury induced by HIV-1 Tat *in vitro* (Bertrand et al., 2014). However, the dose of daidzein (1μM) required for synaptodendritic protection is 100x higher than estrogen (10nM).

During digestion, daidzein is initially broken down into dihydrodaidzein which is further metabolized into either *O*-desmethylangolensin or *cis/trans*-isoflavan-4-ol (Jackson et al., 2011; Lampe, 2009). Finally, *cis/trans*-isoflavn-4-ol is processed into equol. Due to the chiral center at carbon 3 (figure 1A), equol can theoretically exist in either the S or R conformation or a racemic mixture; However, only S-Equol is excreted by humans and animals (Setchell et al., 2005). Equol is a more potent molecule than daidzein, requiring less of the compound to cross the blood-brain barrier in order to achieve the desired effect. Equol effects neuronal mitochondrial function (Yao et al., 2013) and hypothalamic neuronal expression (Patisaul, Todd, Mickens, & Adewale,

2009) *in vivo* indicating that equol can cross the blood-brain barrier at effective concentrations, unlike daidzein.

HIV-1+ cocaine users are also more likely to display neurocognitive deficits (Levine et al., 2006; Nath et al., 2001), however the mechanism producing these neurocognitive and behavioral effects is unclear nor are there any therapeutic agents for these individuals. HIV-1 proteins in combination with cocaine produced enhanced levels of apoptosis (Kendall et al., 2005; Turchan et al., 2001), however the synaptic effects of HIV-1 proteins and cocaine are unknown. The present study uses the quantification of F-actin puncta, a marker of synaptodendritic integrity, to evaluate the effects of HIV-1 Tat and cocaine on the neuronal network at a time point before frank neuronal cell death. Additionally, we evaluate the neuroprotective effects and mechanism of both S- and R-Equol. Our results suggest that HIV-1 Tat and cocaine together produce enhanced synaptodendritic injury which may contribute to the increased incidence of neurocognitive disorders in HIV-1+ cocaine users.

Methods

Ethics Statement

Experiments that utilized animals were in accordance with NIH Guidelines. The Animal Care and Use Committee at the University of South Carolina (animal assurance number: A3049-01) reviewed and approved all animal protocols.

Primary Neuronal Cell Culture

Midbrain and cortical regions were dissected from gestational day 18 Sprague-Dawley rat fetuses (Harlan Laboratories, Indianapolis IN) as previously described (Aksenova et al., 2006; Aksenova et al., 2009; Bertrand et al., 2011). Following dissection tissue was incubated in a solution of 2mg/ml trypsin in Hank's balanced salt solution (HBSS)

buffered with 10mM HEPES (GIBCO Life Technologies, Grand Island, NY) for 10 minutes. Tissue was rinsed with fresh HBSS 3 times and then exposed to soybean trypsin inhibitor (1mg/ml in HBSS) for 2 minutes. Tissue was washed 3 times with HBSS following trypsin inhibitor treatment. For cytomorpholigical studies, cells were distributed to 12 well glass-bottom dishes and 35mm dishes (MatTek Corporation, Ashland, MA) coated with poly-L-lysine following dissociation by trituration. In order to observe distinct second order branching patterns, a low plating density was used (120-140 cells/mm^2). Initial plating media contained Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12; GIBCO) supplemented with 100 ml/L fetal bovine serum (Sigma Chemicals, St. Louis, MO). DMEM/F12 and fetal serum were removed and replaced with an equal amount of serum-free Neurobasal medium after 24 hours. Neurobasal medium had no phenol red and was supplemented with 2% v/v B-27, 2 mM GlutaMAX supplement and 0.5% w/v D-(1) glucose (all ingredients from GIBCO). Cultures were maintained at 37ºC in a 5% CO_2/95% room air-humidified incubator at all times. Fresh Neurobasal medium was supplemented at weekly intervals. Midbrain cultures used for experiments were 21-30 days in vitro (DIV), cortical cultures used for experiments were 14-21 DIV, and both culture types were >85-90% neuronal as determined by MAP-2/GFAP/NucBlue fluorescent staining.

Experimental Treatment

Recombinant Tat 1-86B (LAI/Bru strain of HIV-1 clade B, GenBank accession no. K02013) (Diatheva, Fano, Italy) was added to the serum free growth media (50 nM or 10 nM final concentration) of both cortical and midbrain cell cultures. In experiments where cocaine was included, freshly prepared cocaine stock solution was added to the serum free grown media (1.6 µM final concentration) concurrently with HIV-1 Tat

treatment. Control cultures were treated with an equal amount of vehicle. Cultures were incubated with either 50 nM HIV-1 Tat, 10 nM HIV-1 Tat, 1.6 µM cocaine, or HIV-1 Tat (10 nM) + cocaine for 24 hours prior to fixation.

Cortical and midbrain cell cultures were treated with S-equol (SE; final concentration 33 nM or 50 nM; ≥98.5% purity; Cayman Chemical, Ann Arbor, MI),R-equol (RE; final concentration 33 nM or 50 nM; ≥98.5% purity; Cayman Chemical Ann Arbor, MI), tamoxifen (TMX; selective estrogen receptor modulator; final concentration 100 nM; Tamoxifen citrate; Tocris Bioscience, Ellisville, MD), 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrmidin-3-yl)phenol (PHTPP; estrogen receptor beta antagonist; final concentration 100 nM; Tocris Bioscience, Ellisville MD), 1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride (MPP; estrogen receptor alpha antagonist; final concentration 100 nM; Tocris Bioscience, Ellisville, MD), and (3a*S**,*4R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (G15; membrane estrogen receptor antagonist; final concentration 100 nM; Tocris Bioscience, Ellisville MD). TMX, PHTPP, G15, SE and RE were initially dissolved in DMSO and then diluted in PBS prior to treatment. MPP was initially dissolved in water, and diluted immediately prior to treatment. For protection studies cells were treated with SE or RE (33 nM or 50 nM) 24 hours prior to treatment with 50 nM Tat or 10 nM Tat and 1.6 µM cocaine. In order to elucidate the estrogenic mechanism underlying SE and RE neuroprotection, cells were treated with TMX, PHTPP, MPP, or G15 for 1 hour prior to treatment with SE or RE (50 nM).

Studies examining the plasma levels of S-Equol following daidzein supplementation have found individuals with plasma concentrations of 8-20 ng/ml (See

review: Jackson et al., 2011). The present study chose a low dose of S-Equol (33 nM) and a mid-range dose of equol (50 nM), which are equivalent to plasma concentrations observed in humans following supplementation (8 ng/ml and 12 ng/ml, respectively).

Fluorescent labeling and immunocytochemistry

Primary cortical and midbrain cell cultures were labeled using immunofluorescent techniques carried out in 12-well glass bottom and single 35 mm glass bottom cell culture dishes (MatTek Corporation, Ashland, MA). A modified protocol for filamentous actin (F-actin) staining was used to visualize F-actin puncta (Invitrogen Life Technologies, Grand Island NY). Briefly, primary neuronal cultures were fixed using 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with the F-actin specific probe phalloidin (1:40; AlexaFluor 488) for 20 minutes (Invitrogen Life Technologies, Grand Island NY). In order to prevent nonspecific binding, cells were incubated with 10% normal horse serum in PBS for 2 hours. Next, cells were co-labeled with chicken polyclonal anti-MAP2 (1:1000) (Abcam, Cambridge MA). Goat anti-chicken IgG conjugated with Alexa Red 594 (1:500; Invitrogen Life Technologies, Grand Island NY) NucBlue (Molecular Probes Life Technologies, Grand Island NY), a Hoescht dye, was used to directly identify cell nuclei.

F-actin Puncta

A Nikon Eclipse TE2000-E inverted fluorescent computer controlled microscope (20X objective, 1600 x 1200 px image size, 0.17 µm/px image resolution at 1X zoom) in conjuction with an attached higher resolution CCD camera was used to obtain images of neurons co-labeled with F-actin (green)/MAP-2 (red)/NucBlue (blue) neurons. NIS-Elements software package (Nikon Instruments, Melville, NY, USA) was used to select analyze 3-5 separate neurons with clearly identifiable dendritic arbors and normal nuclear

morphology per well. Saving of images resulted in an inversion of colors, resulting in green F-actin, blue MAP-2, and red NucBlue staining (Figure 4.3). F-actin puncta were identified in several (3-4) second order dendritic fragments (15-75 µm) for the selected neurons. Neurites were required to have continuous MAP-2 immunofluorescence and normal nuclear morphology to qualify for analysis. F-actin structures included fine filopodia, spine protrusions, and patches, while growth cones (F-actin rich structures found at the most distal end of the dendritic terminus) were excluded (Bertrand et al., 2013).

F-actin puncta were detected and quantified as previously described (Bertrand et al., 2014; Bertrand et al., 2013). Briefly, background fluorescence from the 488 (green phalloidin labeled F-actin) channel was subtracted, identified as low fluorescence of the non-synaptic dendritic shaft (20-50au). Clearly visible F-actin rich structures (fine filopodia, spine protrusions, and patches) were manually counted and calculated as number of F-actin labeled puncta per 10 µm.

A trained independent observer, blinded to experimental treatment, quantified puncta from identically processed images, with a very high correlation (r^2=0.90), indicating that the green F-actin puncta were readily identified.

Statistical Analysis

Statistical comparisons were made using ANOVA techniques, and Tukey's multiple comparison tests were used to determine specific treatment effects. Pearson's product-moment correlation coefficient was calculated to verify inter-rater reliability. Comparisons and correlations were calculated using SPSS version 22 (IBM Corporation,

Armonk NY). Data represents mean values ± standard error of the mean (SEM). Significant differences were set at $p \leq 0.05$.

Results

S- and R-Equol prevent HIV-1 Tat 1-86 induced synaptodendritic injury

Primary midbrain cultures were pre-treated with either R-Equol (4.1B; 33 nM or 50 nM) or S-Equol (4.1C; 33 nM or 50 nM) for 24 hours prior to incubation with 50 nM HIV-1 Tat 1-86 (24 hours) (Figure 4.1). There was a significant main effect of treatment $F(1,22)=9.4$, $p \leq 0.001$, with 50 nM HIV-1 Tat 1-86 producing a significant reduction in puncta ($p \leq 0.001$). Pre-treatment with 50 nM R-Equol ($p \leq 0.01$), but not 33 nM R-Equol, prevented significant loss of F-actin puncta following incubation with 50 nM HIV-1 Tat (Figure 4.1B).Pre-treatment with either 33 nM S-Equol ($p \leq 0.05$) or 50 nM S-Equol ($p \leq 0.001$) prevented significant HIV-1 Tat 1-86 induced loss of F-actin puncta (Figure 4.1C). RE and SE treatment did not produce significant damage or proliferation of F-actin puncta alone (Figure 4.1B,C). RE or SE treated Tat (50 nM) treated cultures were not statistically different from vehicle treated controls.

SE and RE act through an estrogen mediated mechanism to prevent HIV-1 Tat 1-86 induced loss of F-actin puncta

In order to elucidate a potential mechanism underlying the neuroprotective effects of RE and SE, primary midbrain neuronal cultures were treated with tamoxifen (TMX), a potent estrogen receptor antagonist, one hour prior to treatment with 50nM SE or RE. Cultures were then incubated with HIV-1 Tat 1-86 (50 nM) for 24 hours. There was a significant effect of treatment $F(3,23)=9.6$, ($p \leq 0.001$). Pre-treatment with TMX prior to SE ($p \leq 0.02$)

and RE ($p \leq 0.02$) incubation resulted in significant damage to the neuronal network relative to control cultures, but were not significantly different from HIV-1 Tat (50 nM) treated cultures suggesting that RE and SE act through an ER dependent mechanism (figure 4.1D).

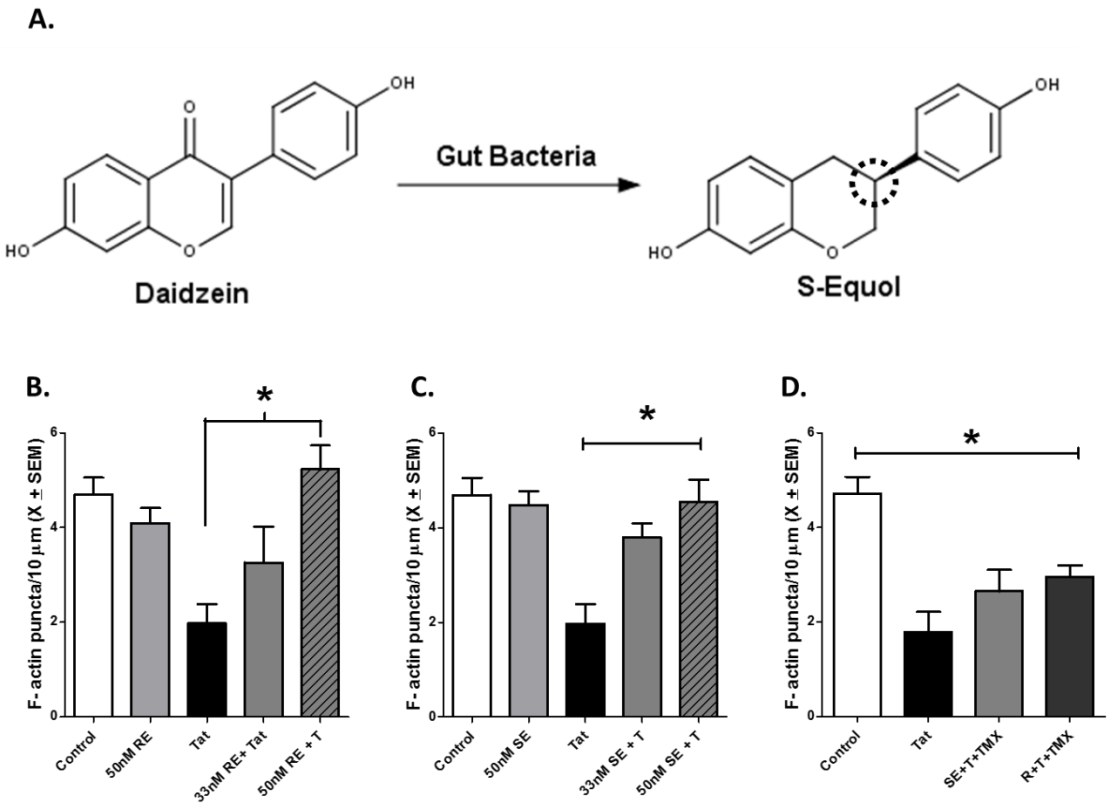

Figure 4.1 SE and RE prevent significant synaptodendritic damage induced by a toxic (50 nM) dose of HIV-1 Tat 1-86 in an estrogen receptor mediated mechanism. **A**. Metabolism of daidzein to S-Equol. The circle represents a chiral center on carbon 3 where the theoretical conformational change would occur to produce R-Equol. However, only S-Equol is naturally produced by mammalian gut bacteria. **B.** A moderate dose of R-Equol (50 nM), but not a low dose (33 nM), prevents significant synaptodendritic injury induced by HIV-1 Tat 1-86 (50 nM; $p \leq 0.01$). **C**. Both the low (33 nM) and moderate (50 nM) doses of S-Equol prevent HIV-1 Tat 1-86 induced synaptodendritic injury ($p \leq 0.05$ and $p \leq 0.001$, respectively). **D.** One hour incubation with TMX blocked the neuroprotective effects of RE and SE (50 nM) against a toxic dose of HIV-1 Tat 1-86 (50 nM) in primary midbrain cultures. Data represents mean values ± SEM, *$p \leq 0.05$ compared to HIV-1 Tat treated cultures.

HIV-1 Tat and cocaine interact to produce synaptodendritic injury in primary midbrain and cortical cell cultures

In order to examine the effects of HIV-1 Tat and cocaine on synaptodendritic integrity, a sub-toxic dose of HIV-1 Tat 1-86 (10 nM) and a physiologically relevant dose of cocaine (1.6 µM) were simultaneously added to primary midbrain and primary cortical cell cultures and incubated for 24 hours. As shown in figure 4.2 (A,D), there was a significant interaction between HIV-1 Tat and cocaine $F(1,123)=16.4$, ($p\leq0.001$) but not a significant main effect of either HIV-1 Tat or cocaine individually on synaptodendritic integrity when compared to controls. There was not a significant main effect of region $F(1,123)=3.4$, $p>0.05$.

SE and RE prevent interactive HIV-1 Tat + cocaine synaptodendritic damage

RE and SE neuroprotection against HIV-1 Tat (50 nM) induced damage was an ER dependent mechanism (figure 4.1). Thus, we hypothesized that treatment with either equol enantiomer would prevent HIV-1 Tat+cocaine induced puncta loss using a similar or identical mechanism. In order to evaluate our hypothesis, primary cortical and midbrain cultures were pre-treated with 50 nM RE or SE for 24 hours prior to a 24 hour incubation with Tat 1-86 (10 nM) and cocaine (1.6 µM). There was a significant main effect of treatment $F(5,123)=7.6$ $p\leq0.001$ (Figure 4.2 B,E), but not a significant effect of region ($p>0.05$). Using a Tukey post hoc analysis it was determined that Tat + cocaine cultures pretreated with RE or SE had significantly more puncta relative to Tat + cocaine cultures, ($p\leq0.001$ for both conditions) and were not significantly different than vehicle treated controls ($p>0.1$ for both conditions).

SE and RE act through an ERβ mediated mechanism to prevent HIV-1 Tat+cocaine induced synaptodendritic injury

Next, primary cortical and midbrain cultures were treated with TMX, an estrogen receptor antagonist, for one hour prior to treatment with RE or SE. Following a 24 hour incubation with Tat + cocaine, cultures were fixed and F-actin puncta assessed. There was a significant main effect of treatment $F(8,104)=4.3$, $p\leq0.001$, with TMX significantly blocking RE or SE mediated neuroprotection $p\leq0.01$ (Figure 4.2 C,F).

Finally, cortical cultures were treated with one of 3 selective ER subtype antagonists, MPP (ERα), PHTPP (ERβ), or GPR30 (G15) for 1 hour prior to treatment with RE or SE in order to identify the specific ER subtype required for equol mediated neuroprotection (Figure 4.3 A,B). There was a significant main effect of antagonist treatment $F(3, 134)=11.2$, $p\leq0.001$. Both MPP and G15 treated cultures were not different from vehicle treated controls ($p>0.05$), however PHTPP treated cultures had a significant loss of F-actin puncta when compared to controls ($p\leq0.05$) and were not significantly different from Tat + cocaine treated cultures ($p>0.05$). More specifically, only the ERβ antagonist PHTPP significantly prevented RE or SE mediated neuroprotection ($p\leq0.001$), indicative of an ERβ dependent mechanism.

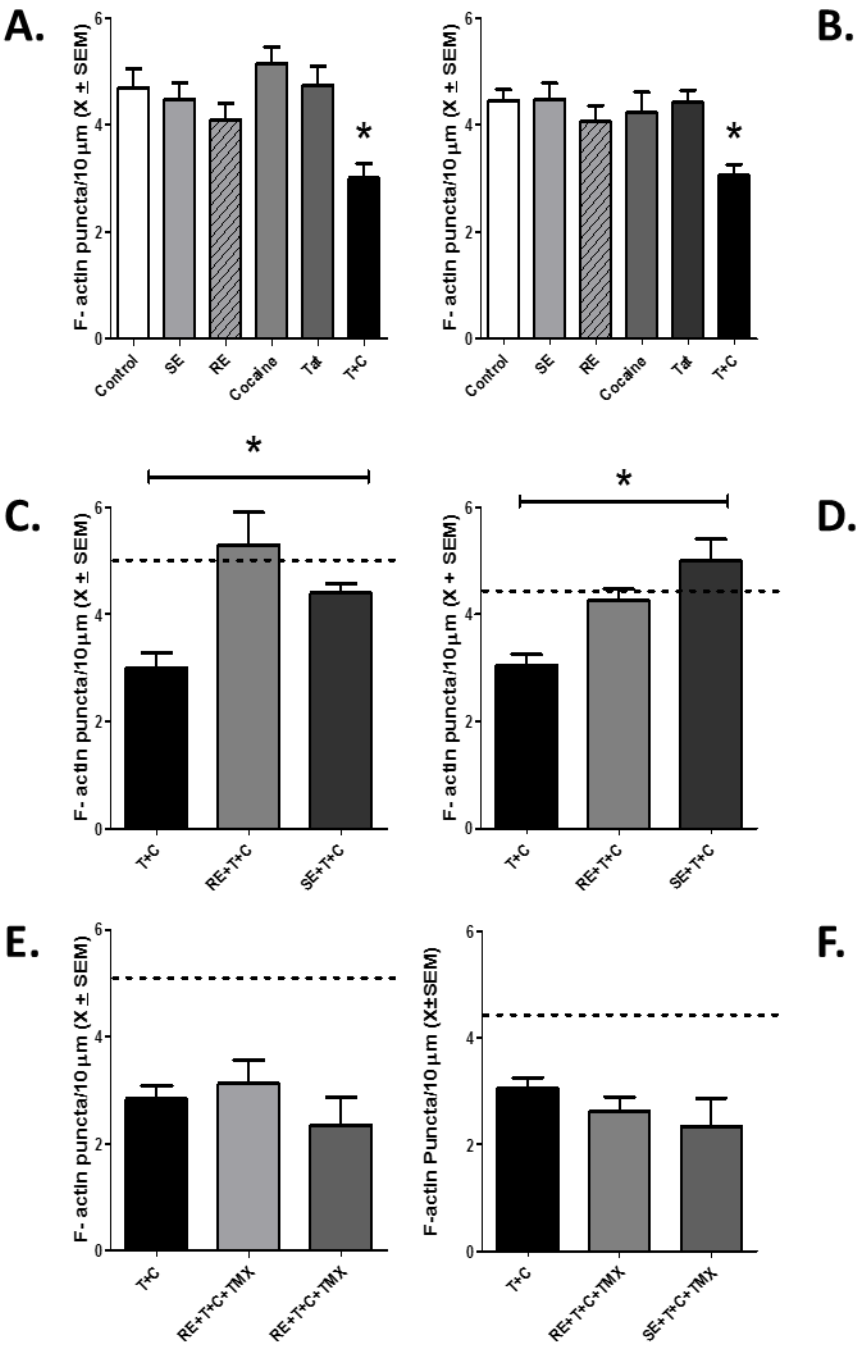

Figure 4.2 Treatment with HIV-1 Tat 1-86 (10 nM) + cocaine (1.6 µM) produces significant synaptodendritic injury in cortical and midbrain cultures **A**. Treatment with SE, RE, HIV-1 Tat 1-86 (10 nM), or cocaine (1.6 µM) do not significantly alter density of F-actin puncta compared to controls in primary midbrain neuronal cultures. Treatment with both HIV-1 Tat (10 nM) and cocaine (1.6 µM) induces a significant loss of F-actin puncta ($p \leq 0.05$). **B.** Treatment with SE, RE, HIV-1 Tat 1-86 (10 nM), or cocaine (1.6

μM) do not significantly alter synaptodendritic integrity compared to controls in primary cortical neuronal cultures. Treatment with HIV-1 Tat (10 nM) and cocaine (1.6 μM) produces significant synaptodendritic injury ($p \leq 0.05$). **C.** Pre-treatment with RE or SE (both 50 nM) prevents significant synaptodendritic damage induced by Tat + cocaine in primary midbrain cultures and are not significantly different from control cultures. **D.** Pre-treatment with RE or SE (both 50 nM) prevents Tat+cocaine induced loss of F-actin puncta ($p \leq 0.001$) in primary cortical cell cultures and are not significantly different from vehicle treated controls. **E.** Treatment with TMX (100 nM) for 1 hour prior to RE or SE treatment in primary midbrain cultures prevents the neuroprotective effects of RE and SE suggesting an estrogen receptor mediated mechanism. **F**. Treatment with TMX (100 nM) for 1 hour prior to RE or SE treatment blocks RE and SE neuroprotective effects indicating an estrogen receptor mediated mechanism in primary cortical cell cultures. Data represents mean values ± SEM, *$p \leq 0.05$ compared to HIV-1 Tat + cocaine (C,D) or vehicle treated controls (A,B).

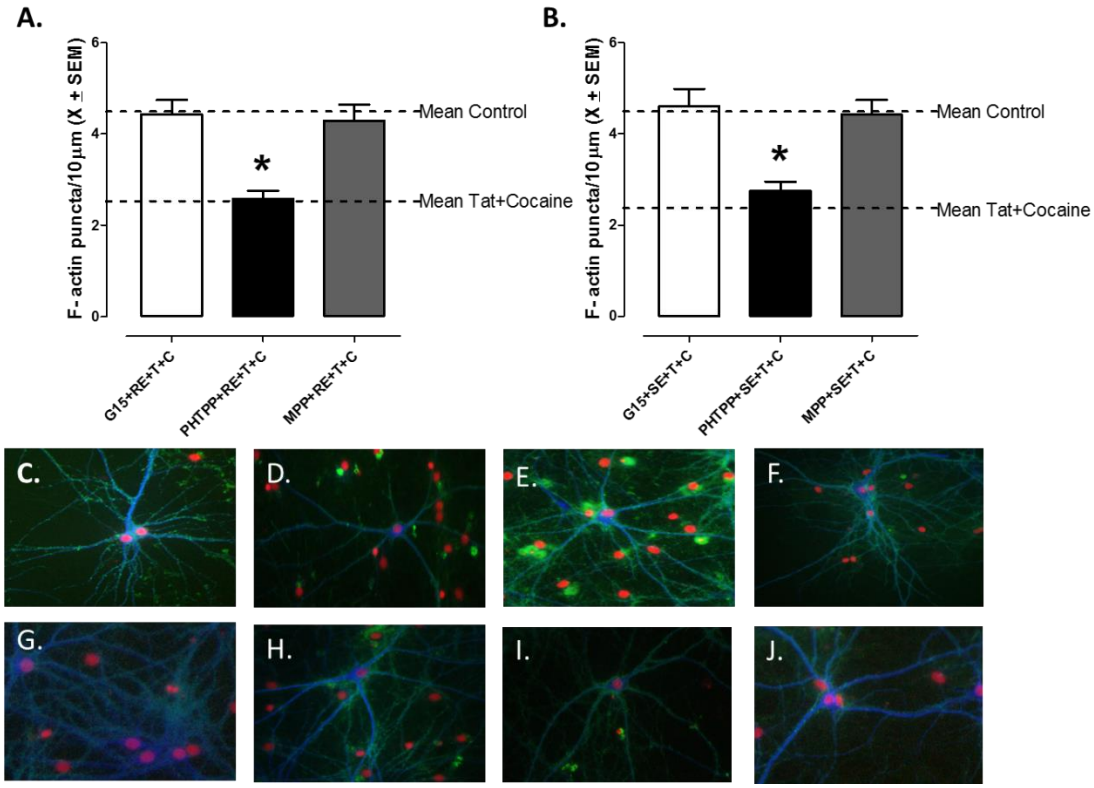

Figure 4.3 RE and SE prevent Tat + cocaine induce synaptodendritic injury in an ERβ dependent mechanism. Primary cortical neurons were pre-treated with one of 3 selective estrogen receptor antagonists, **A.** Mean (±SEM) puncta/10 μm following treatment with all 3 ER antagonists and RE. PHTPP (beta selective antagonist) treatment prior to RE pretreatment precluded RE mediated protection resulting in significant synaptodendritic damage compared to controls. **B.** Mean (±SEM) puncta/10 μm following treatment with all 3 ER antagonists and SE. PHTPP treatment prior to SE pre-treatment resulted in significant synaptodendritic damage compared to controls. **C-J.** Representative images.

Vehicle treated (**C**), HIV-1 Tat (10 nM; **G**), and cocaine treated (**H**) controls and SE(**E**)/RE(**F**) pre-treated cultures demonstrate robust staining with F-actin(green) and MAP-2 (blue) neurites with complex branching patterns and a lack of aberrant features such as bundling or beading. PHTPP pre-treated SE(**I**)/RE(**J**) and Tat + Cocaine treated cultures illustrate the effects of synaptodendritic damage; there is a lack of fine network in the background of the image in addition to a significantly reduced F-actin stain and stunted dendritic branching. Data represents mean values ± SEM, *$p \leq 0.05$ compared to controls.

Discussion

Neurocognitive deficits are more prevalent among HIV-1+ positive drug users (Devlin et al., 2012; Nath, 2002; Weber et al., 2013; Levine et al., 2006). Cocaine is one of the most commonly used drugs in HIV-1+ individuals (Cook et al., 2007; Korthuis et al., 2008), and has been shown to augment the neurotoxicity of HIV-1 proteins *in vitro* (Kendall et al., 2005; Turchan et al., 2001; Aksenov et al., 2006). Although neuronal cell death is seen in HAND (Adle-Biassette et al., 1995; Adle-Biassette et al., 1999; Li, Galey, Mattson, & Nath, 2005; Mattson et al., 2005), synaptic dysfunction and damage are more predictive of neurocognitive status (Adle-Biassette et al., 1999; Ellis et al., 2007). HIV-1 Tat + cocaine treatment resulted in a significant reduction in F-actin rich puncta, indicating aberrant neuronal function. Pre-treatment with RE or SE prevented HIV-1 Tat + cocaine induced loss of puncta via an ERβ dependent mechanism, suggesting early treatment with RE or SE may prevent further neuronal damage or loss, potentially slowing or stopping the progression of HAND.

Cocaine potentiates the effects of HIV-1 Tat, as the Tat dose used in the combination studies was not damaging alone. The dose of HIV-1 Tat chosen was 4x lower than concentrations previously shown to lack neurotoxic properties in primary neuronal cell culture (Kendall et al., 2005; Turchan et al., 2001). The dose of cocaine

used was within the physiological levels observed in IV cocaine users (Evans, Cone, & Henningfield, 1996) and is well below the range known to produce neuronal toxicity *in vitro* (Nassogne, Evrard, & Courtoy, 1995). However, HIV-1 Tat + cocaine produce synaptodendritic injury prior at a time point prior to cell death, suggesting that cocaine potentiates the effects of HIV-1 Tat. Cocaine enhances monocyte migration across the blood brain barrier and disrupts intercellular junctions (Fiala et al., 2005; Fiala et al., 1998), increasing the number of virally infected cells in the brain. Additionally, cocaine increases viral replication in monocytes (Peterson et al., 1991), macrophages (Roth et al., 2002), and astrocytes (Reynolds et al., 2006) suggesting that cocaine may have powerful intracellular interactions with HIV-1 Tat. HIV-1 Tat and cocaine both inhibit dopamine transporter (DAT) function (Midde et al., 2013; Ritz & Kuhar, 1993; Ferris, Frederick-Duus, Fadel, Mactutus, & Booze, 2009b), which may contribute to an increase in oxidative stress (Aksenov et al., 2006) and thus result in the depolymerization of F-actin and a subsequent reduction of F-actin rich puncta.

Non-drug abusing HIV-1+ individuals have significant levels of neuronal damage (Moore et al., 2006; Maragos et al., 2003; Adle-Biassette et al., 1999; Masliah et al., 1997) suggesting that extracellular HIV-1 protein levels are neurotoxic and not sub-toxic as used in the present study. However, by using a sub-toxic dose of HIV-1 Tat we demonstrate that even a low dose of a singular protein in combination with a physiologically relevant dose of cocaine result in significant synaptodendritic insult. Our results suggest that the synaptodendritic damage observed in an HIV-1+ cocaine user would 1. Have more severe synaptodendritic damage than a non-cocaine user, and 2. Be more severe than what is demonstrated by the present *in vitro* study. Identification of

synaptodendritic injury is important because it occurs prior to cell death (Bertrand et al., 2013) and is potentially reversible (Bertrand et al., 2014; Kim et al., 2008a).

S-equol prevents HIV-1 Tat induced synaptodendritic injury at a lower concentration (33 nM) than R-Equol (50 nM), demonstrating that the neuroprotective properties of equol are specific to the conformation of the molecule. Equol is the active metabolite of the soy derived phytoestrogen daidzein (Setchell & Cassidy, 1999; Jackson et al., 2011), and has been proposed to be the driving force behind the cognitive health benefits of soy products (Jackson et al., 2011; Lampe, 2009; Setchell & Cassidy, 1999). Daidzein is metabolized into equol via two distinct steps by various bacteria found in the gut of animals and humans (Jackson et al., 2011; Setchell et al., 2005). Importantly, only S-Equol is naturally produced by animal and human bacterial flora in the gut (Setchell et al., 2005). In humans, daidzein supplementation results in plasma concentrations of 8-20ng/ml S-equol (See review: Jackson et al., 2011). Presently, a low dose of S- and R-equol (33 nM) and a mid-range dose of equol (50 nM), which are equivalent to plasma concentrations observed in humans following supplementation (8 ng/ml and 12 ng/ml, respectively). Both enantiomers achieve full neuroprotection at physiologically relevant doses and with a concentration that is at least 20x lower than that of daidzein required for full synaptodendritic protection (Bertrand et al., 2014). Introduction of daidzein into the medium of neuronal cell cultures exposed to a toxic dose of HIV-1 Tat results in a restoration of the F-actin rich network in an estrogen receptor dependent manner (Bertrand et al., 2014), combined with the present study this suggests that equol may also aid in the restoration of the fine network.

Both equol enantiomers prevent HIV-1 Tat-induced (50nM) and HIV-1 Tat (10nM) + cocaine induced synaptodendritic damage. TMX treatment blocked the neuroprotective actions of both RE and SE, indicating that equol mediated neuroprotection was via an estrogen receptor dependent mechanism. S-Equol is a potent ER agonist, with preferential binding to ERβ over ERα (Setchell et al., 2005; Jackson et al., 2011; Lampe, 2009). In order to determine the mechanism of action of both S- and R-Equol for neuroprotection against HIV-1 Tat and cocaine, three estrogen receptor subtypes α, β, and GPR30 were examined. MPP, PHTPP, and G15 (ERα, ERβ, and GPR30 antagonists, respectively) were employed to determine which ER subtype mediated the neuroprotective effects of S- and R-equol. Only PHTPP precluded the neuroprotective effects of S- and R-Equol, indicating that both enantiomers provide neuroprotection via an ERβ dependent mechanism. Importantly, identical concentrations of equol (50nM) were used to achieve significant neuroprotection, suggesting that this treatment may be beneficial for both HAND and HAND comorbid with drug abuse.

Treatment with 17β-estradiol mitigates interactions between HIV-1 protein and illicit drugs resulting in a prevention of neuronal cell death (Kendall et al., 2005; Turchan et al., 2001). However, ERα and ERβ activation play differential roles in protection of CNS cells in HIV-1. Treatment with 17β-estradiol suppresses Tat enhancement of LTR promoter activity in astrocytes (Wilson et al., 2006). Meanwhile over expression of ERα prevents suppression of Tat-LTR promoter activity in astrocytes (Heron et al., 2009), although the specific effects of ERβ in this process are not well understood. Action at the ERβ prevents HIV-1 Tat induced apoptosis (Adams et al., 2010; Adams et al., 2012). Additionally, activation of the ERα subtype may lead to unwanted side effects such as

expression of secondary sex characteristics or cancer, suggesting that an ERβ specific compound, like equol, would be beneficial for preventing neuronal damage and potentially controlling Tat-LTR interactions resulting in a decrease or prevention of viral replication.

The gut-brain axis is a new area of study which recognizes the bidirectional communication between the gut microbiota and behavior (Cryan & Dinan, 2012; Foster & McVey Neufeld, 2013). Anxiety-like and depressive-like behaviors have been treated with probiotics in rats and mice (Neufeld, Kang, Bienenstock, & Foster, 2011a; Neufeld, Kang, Bienenstock, & Foster, 2011b), and have been suggested as a treatment for IBS, a gastrointestinal disorder linked to increased amounts of stress (Clarke, Cryan, Dinan, & Quigley, 2012). Equol is produced by the gut microbiota in humans and animals, but not all humans are equol producers. Up to 80% of individuals in China, Japan, and Korea are capable of producing equol (Fujimoto et al., 2008; Morton, Arisaka, Miyake, Morgan, & Evans, 2002), however only 25% of the population that consumes a Western diet are equol producers (Lampe, Karr, Hutchins, & Slavin, 1998). HIV-1 is a pandemic, however there are higher incidences of HAND in individuals in North America and Europe as opposed to Sub-Saharan Africa and Asian populations (Satishchandra et al., 2000). These regional differences have been contributed to differences in HIV-1 strain, however some variability could be due to differences in gut microbiota. It is currently unknown how HIV-1 may affect the gut microbiota, however it may play an important role in mediating disease progression as well as neurocognitive correlates of HIV-1. The ability of the gut metabolite equol to alter synaptodendritic injury induced by HIV-1 Tat and HIV-1

Tat+cocaine suggests that the gut may play an important role in modulating CNS health in HIV-1.

Our results indicate that HIV-1 protein Tat in combination with cocaine results in significant damage to the synaptic network which may play a role in the increased prevalence of HAND in cocaine users (Nath et al., 2001; Purohit et al., 2011). Cognitive function in HAND is closely correlated with synaptodendritic injury (Ellis et al., 2007), and our results represent a potential culprit for the increased prevalence of HAND in HIV-1+ cocaine users (Nath et al., 2001; Gill & Kolson, 2014; Martin-Thormeyer & Paul, 2009). Importantly, synaptodendritic injury occurs prior to overt cell death (Bertrand et al., 2014; Kim, Martemyanov, & Thayer, 2008b; Bertrand et al., 2013) and is believed to be reversible (Bertrand et al., 2014; Kim et al., 2008a), emphasizing the importance of early detection and treatment. We also demonstrate that both equol enantiomers prevent Tat+cocaine induced synaptotoxicity through an ERβ dependent mechanism. Action specifically at ERβ may limit unwanted side-effects, yet still promote neuroprotection similar to the effects of 17β-estradiol (Adams et al., 2010; Kendall et al., 2005; Turchan et al., 2001; Simpkins, Singh, Brock, & Etgen, 2012; Simpkins & Singh, 2008). Equol is more potent than the parent compound daidzein, and works via a similar, if not identical, mechanism (Adams et al., 2012), suggesting that treatment with S- or R-Equol may promote network restoration following HIV-1 Tat or Tat+cocaine insult at similar or enhanced rates compared to daidzein (Bertrand et al., 2014). The present study provides further evidence that ERβ is a novel target to prevent synaptodendritic injury and that gut metabolites, like equol, may play an important role in treating HIV-1 associated neuronal injury.

CHAPTER 5

HIV-1 DISRUPTS MOTIVATION VIA DOPAMINE TRANSPORTER DYSREGULATION[4]

[4] SJ Bertrand, CF Mactutus, SB Harrod, LM Moran, RM Booze. Submitted to *Journal of Neuroscience* March 16, 2015.

Introduction

HIV-1 infection and drugs of abuse may exert synergistic effects on reward processes and motivation (Carey et al., 2006; Rippeth et al., 2004; Chang et al., 2008; Ferris, Mactutus, & Booze, 2008; Nath et al., 2001). Motivation is defined as a fundamental state that allows an animal to regulate its internal and external environments through the organization of behavior (Salamone & Correa, 2012). Goal-directed behavior, which encompasses Pavlovian and operant conditioning processes, is one example (Rescorla, 1987). Once learned, goal-directed behavior is maintained by motivational and cognitive processes, such as the animals' homeostatic state, an expectation about the reinforcer, the current value of the reinforcer, environmental cues, and other learning phenomena, such as generalization and discrimination (Spear & Riccio, 1994; Koob, 2009). Although the construct of motivation is widely used, it has received little attention in HIV-1/AIDS research.

Interestingly, neural systems that mediate the motivational features of goal-directed behavior (Everitt and Robbins, 2013) are damaged by HIV-1 (Everitt & Robbins, 2013; Purohit et al., 2011; Nath et al., 2000). HIV-1+ individuals exhibit high HIV-1 viral titers in the caudate (Kumar et al., 2007; Kumar et al., 2009b) and extensive atrophy in the caudate-putamen and NAc are reported in HIV-1 infected patients exhibiting cognitive impairment (Paul et al., 2002; Hestad et al., 1993; Dal Pan et al., 1992).

HIV-1+ patient brains show impairments in dopaminergic cell bodies (Silvers et al., 2006; Itoh, Mehraein, & Weis, 2000) and loss of synaptic connectivity in the dopaminergic projection pathways (Gelman, Spencer, Holzer, III, & Soukup, 2006). Interestingly, dopamine transporter (DAT) proteins are significantly reduced in cognitively impaired HIV-1 patients (Wang et al., 2004) and DAT function is reduced further in HIV-1+ cocaine abusers (Chang et al., 2008). Thus, the DA systems in the human brain appear particularly vulnerable to HIV-1 infection.

The HIV-1 Tg rat incorporates HIV-1 proviral DNA into its genome, without active viremia (Reid et al., 2001). The aviremic state in the HIV-1 Tg rat may be similar to that reported for individuals latently infected with HIV-1, or treated with cART to reduce peripheral viral loads. In the absence of active infection or viral replication, the presence of HIV-1 proviral DNA may induce production of HIV-1 proteins. Brains from HIV-1+ patients without HIV-1 viral RNA/p24 (i.e. latent infections), show decreases in synaptodendritic markers (Desplats et al., 2013) and the presence of HIV-1 Tat protein in the cerebral spinal fluid (Johnson et al., 2013). The HIV-1 Tat protein directly inhibits DAT via specific Tat protein:DAT protein interactions (Zhu et al., 2009; Midde et al., 2013). Although the DAT mediates many of the reinforcing aspects of cocaine, and HIV-1 proteins interact with DAT (Wallace et al., 2006), it is unknown how chronic, low-levels of HIV-1 proteins (i.e. Tat) alters the motivational and neurochemical responses to cocaine.

We used the HIV-1 transgenic (Tg) rat to examine alterations in motivational processes and dopaminergic neurochemical correlates in the striatum and prefrontal cortex. Specifically, we investigated goal-directed behavior, using self-administration of

IV cocaine or sucrose as reinforcement, and neurochemical alterations in the DAT. These findings could elucidate specific interactions between cocaine abuse and HIV-1 and foster therapeutic strategies to treat this comorbidity in vulnerable populations.

Methods

Animals

Female HIV-1 Transgenic (Tg) rats (*n*=14) and female F344/NHsd control rats (*n*=15) were procured at approximately 60 days of age from Harlan Laboratories, Inc. (Indianapolis, IN, USA). The generation of the HIV-1 Tg rats has been previously described (Reid et al., 2001). In brief, the hemizygous HIV-1 Tg animals were produced using a construct derived from an infectious provirus (pNL4-3) after deletion of a 3.1 kb *Sph1-Bal1* fragment. This fragment overlaps the *gag* and *pol* genes (pNL4-3d1443). Thus, the HIV-1 Tg rats do not produce gag or pol proteins, which are necessary for viral structures (Peng et al., 2010), thus, these animals are non-infectious. Viral proteins, such as Tat and gp120, are produced under the control of the native HIV-LTR in disease appropriate mononuclear phagocytes and astrocytes, but not in neurons, similar to that in human neuroAIDS (Royal et al., 2012). HIV-1 Tg young adult female rats are generally healthy (Roscoe et al., 2014), can perform operant behavioral tasks (Moran et al., 2014), and grow at similar rates as F344 controls (Moran et al., 2013).

All rats were ovariectomized (OVX) at Harlan Laboratories prior to arrival at the University of South Carolina. Ovariectomy was performed as estradiol potentiates the reinforcing efficacy of cocaine (Hu, Crombag, Robinson, & Becker, 2004), protects against HIV-1 mediated neuronal damage *in vitro* (Bertrand et al., 2014; Adams et al., 2010; Heron et al., 2009), and suppresses HIV-1 transcription (Cabrera-Munoz,

Hernandez-Hernandez, & Camacho-Arroyo, 2012; Szotek, Narasipura, & Al-Harthi, 2013). In addition, rats were fed a minimal phytoestrogen diet (≤20 ppm of phytoestrogen; Teklad 2020X Global Rodent Diet; Harlan Laboratories, Inc., Indianapolis, IN). Phytoestrogens are plant-derived compounds that are structurally similar to estrogen, have estrogenic effects, and are found in soybean and alfalfa meal (Lephart et al., 2005; Setchell & Cassidy, 1999). Soy and alfalfa meal are typical components of rodent chow; standard rodent chow typically contains 200-500 ppm (Harlan Laboratories, Inc., USA). Food and water were provided *ad libitum* throughout the experiment, unless otherwise specified. The targeted conditions for maintenance of the animal vivarium were a temperature of 20±2˚C, 50±10% relative humidity and a 12L:12D cycle with lights on at 0700h. The Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina (animal assurance number: A3049-01) approved this research.

Apparatus

Sucrose preference experiment. Animals received access to water or sucrose solutions in 100 ml graduated cylinders equipped with a # 6.5 rubber stopper and 2.5'' straight drinking tube (OT-100; Ancare, Bellmore, NY, USA) that was placed on the testing cage. Animals were tested in clear polycarbonate cages containing standard rodent bedding. Five 100 ml graduated cylinders, containing four sucrose solutions or water, were fitted to the cage-top of the rats' testing cage. The testing cages were located on a single cage rack and the experiment was conducted in the colony room.

Operant conditioning experiments. The individual operant chambers were within sound-attenuating enclosures. The front and back panels of the operant chambers (ENV-

008; Med-Associates, St. Albans, VT, USA) were stainless steel panels, the sides and top of the chamber were constructed of polycarbonate. The front panel of the chamber housed a panel containing a receptacle that allows a recessed 0.01 cc dipper cup (ENV-202C) to deliver a solution through a 5-cm x 5 cm opening following completion of a response requirement (ENV 202M-S). Two retractable metal levers (ENV-112BM) on either side of the receptacle were located 7.3 cm above a metal grid floor. These were "active" levers (responding on them could result in reinforcement). A 28-V white cue light, 3 cm in diameter, was located above each active response lever but never illuminated. An infrared sensor (ENV-254-CB) was used to detect head entries into the receptacle. An additional non-retractable lever was positioned on the back wall of the chamber and a 28-V house light was located above the lever. This was the "inactive" lever and there were no programmed response-reinforcer contingencies with the lever. During cocaine self-administration tests a syringe pump (PHM-100) was used to deliver intravenous drug infusions through a water-tight swivel (Instech 375/22ps 22GA; Instech Laboratories, Inc., Plymouth Meeting, PA), which was connected to the back mount of the animal using Tygon tubing (ID, 0.020 IN; OD, 0.060 IN) enclosed by a stainless steel tether (Camcaths, Cambridgeshire, Great Britain). The pump infusion times were calculated by a Med-PC computer program according to the animal's daily bodyweight.

Drugs

Cocaine hydrochloride (Sigma-Aldrich Pharmaceuticals, St. Louis, MO) was weighed as salt base and was dissolved in physiological saline (0.9%; Hospira, Inc. Lake Forest, IL). Cocaine and sucrose solutions were prepared fresh prior to the start of each session to prevent significant hydrolysis of cocaine and to ensure that each group received similar exposure to either solution prior to each operant test. Heparin was

purchased from APP Pharmaceuticals (Schaumburg, IL.), Butorphenol (Dolorex) from Merck Animal Health (Millsboro, DE), Sevofluorane, USP from Baxter (Deerfield, IL), and Gentamicin sulfate from VEDCO (Saint Joseph, MO).

Synaptosomal [^{3}H]DA Uptake Assay and [^{3}H]WIN35,428 Binding

The radioligands [^{3}H]WIN 35,428 (-)-3β-(4-flurophentl)-tropan-2β-carboxylic acid methyl ester tartrate - specific activity, 85 Ci/mmol, and [^{3}H]DA (3,4-ethyl-2[*N*-^{3}H] dihydroxyphenylethylamine - specific activity, 31 Ci/mmol, were purchased from PerkinElmer Life Sciences (Boston, MA, USA). D-Glucose was purchased from Aldrich Chemical (Milwaukee, WI). L-Ascorbic acid, bovine serum albumin, pyrocatechol, α-D-glucose, HEPES, nomifensine maleate, desipramine hydrochloride, paroxetine hydrochlorine, pargyline hydrochloride, and sucrose were purchased from Sigma Aldrich (St. Louis, MO). Other chemicals were obtained from Thermo Fisher Scientific (USA).

Overall Experimental Design

A two-group between subjects design was employed (HIV-1 Tg vs. F344/NHsd). All animals were successively assessed, trained, and tested for approximately six months, as illustrated in the design table (Table 1).

Experiment 1: Sucrose Taste Preference.

A sucrose taste preference test was conducted to assess HIV-1Tg rats' preference for one of five concentrations of sucrose, i.e., 0, 1, 3, 10, and 30% (w/v), relative to F344 controls. In order to habituate the rats to the novel bottles and stoppers, rats were placed in a clean testing cage with access to five graduated cylinders that contained water. This procedure occurred for 30 min per day for 3 consecutive days. The sucrose preference tests were conducted for five consecutive days following habituation. During testing animals were placed in a clean testing cage and had free access to the sucrose solutions

and water for several hr following testing. To control for a position bias, bottle order was randomized for each rat on the first day of testing and bottles were rotated on each subsequent day using a Latin square procedure so that each concentration was in each location at least once. Bottles were weighed and menisci read to the nearest ml before and after each test to determine the amount of fluid consumed from each bottle.

Experiment 2: Sucrose-Maintained Responding

The animals from Experiment 1 were used in the subsequent experiments, and thus all animals were sucrose experienced. Dipper training and auto-shaping were conducted according to previous research (Harrod et al., 2012; Lacy et al., 2012; Reichel et al., 2008). All rats learned to approach the receptacle and drink from the dipper (dipper training) and subsequently respond for 5% sucrose (w/v) reinforcement (autoshaping). Rats were water restricted for 12-15 hr prior to dipper training, autoshaping, and fixed ratio (FR) training. After completion of the daily operant test, animals were given free access to water in their home cage for 9-12 hr.

Fixed-ratio (FR) schedules of reinforcement provide the reinforcer following a fixed set of responses. In the case of drug reinforcement responding changes if the unit-dose of the self-administered drug is varied; a higher dose would result in fewer responses, although the higher dose is presumably more reinforcing (Yokel & Pickens, 1973; Caine & Koob, 1994a). In order to assess the reinforcing efficacy of a drug, a progressive-ratio (PR) schedule of reinforcement is often used (Richardson & Roberts, 1996). According to the PR schedule, the number of responses required to produce reinforcement is increased following completion of each ratio requirement, eventually resulting in a break point, wherein responding is markedly decreased. Putatively, the higher the break point, the more reinforcing a particular stimulus is considered, and

motivation to attain the reinforcer can be determined (Caine & Koob, 1994b; Harrod, Lacy, & Morgan, 2012a; Lacy, Morgan, & Harrod, 2014; Lacy, Hord, Morgan, & Harrod, 2012a).

Two successive experiments were conducted to investigate acquisition of sucrose-maintained responding. Experiments 2A and 2B investigated sucrose-maintained responding under conditions of water restriction and non-restriction (*ad libitum* water), respectively. In both experiments animals were required to respond for 5% sucrose according to an FR1 schedule of reinforcement. The FR1 schedule requires one response on an active lever to receive the consequent reinforcer; hence, the ratio requirement is fixed. At the beginning of the operant test the active and inactive levers were available. Rats responded for sucrose reinforcement (5%, w/v) on the two active levers during 42 minute operant tests. A single response on an active lever resulted in 4s of access to sucrose. In order to prevent the development of a response side bias, if an animal made five consecutive responses on either one of the active levers, that lever was programmed to retract. If a lever was retracted, the alternative lever was available for responding. Once the asymmetry in responding was balanced the previously retracted lever was once again made available. The use of two active levers was also important from the perspective of training the animals about response variability (Grunow and Neuringer, 2002), especially in light of the subsequent assessment of choice behavior (Experiment 4). Responding on the inactive lever was recorded, but no programmed consequence was presented. Animals were required to meet a criterion of 60 or more reinforcers per test over 3 consecutive days. Once this criterion was met under conditions of water restriction

(2A), the experiment was conducted again under conditions of *ad libitum* water (2B). No further water restriction was used for any subsequent experiment.

Experiment 2C determined the sucrose-maintained responding curves for the F344 or HIV-Tg rats using the FR1 schedule of reinforcement. Testing began after three consecutive days of 60 or more reinforcers using the training concentration of sucrose (5%; FR1). For the tests, one of five sucrose concentrations (1%, 3%, 5%, 10%, and 30%; w/v) was presented, according to a Latin-square procedure. There were six total concentration-response test days and those occurred every other day. Maintenance trials occurred on the non-test days. The training concentration of sucrose (5%) was the reinforcer on the maintenance days. Water was the reinforcer on the sixth and final test day. Water was used primarily as a control condition to assess responding in the absence of sucrose. It was presented as the sixth and last reinforcer for all rats in order to prevent potential extinction learning if water was not reinforcing to the animals (e.g., the absence of reinforcement). No water restriction was used for this experiment.

Experiment 2D determined the sucrose-maintained responding curves for the F344 or HIV-Tg rats using the progressive-ratio (PR) schedule of reinforcement. Animals were experienced with FR schedules and did not require new training in order to progress to the PR phase of the experiment. After three maintenance days (5%; FR1) the schedule of reinforcement was changed to PR. During PR tests meeting the ratio requirement yielded 4-s access to reinforcement. Ratios progressively increased according to an exponential function (Response ratio (rounded to nearest integer) = $[5e^{(\text{injection number} X 0.2)}] - 5$) (Richardson & Roberts, 1996).

Fig. 5.2 shows the values for a number of rats mixed responses across the two active levers, then reinforcement was delivered after the ratio requirement was completed. One of five sucrose concentrations (1%, 3%, 5%, 10%, and 30%; w/v; Latin square procedure) was presented as the reinforcer on the concentration-response test days, which occurred every other day. Maintenance trials, which made 5% sucrose available (FR1), occurred on the non-PR test days. Water was presented as the sixth and last reinforcer for all rats in order to prevent potential extinction learning if water was not reinforcing to the animals. The PR tests were a maximum of 120 min in length. No water restriction was used for this experiment.

Experiment 3: Cocaine-Maintained Responding

Surgery

Following completion of experiment 2D, all animals were implanted with IV catheters. IV catheterization was performed according to established methods (Smith, Lacy, & Strickland, 2014). Anesthesia was induced using 5-7% inhalant sevofluorane and animals were maintained at 3-4% sevofluorane throughout the surgical procedure. Following anesthesia, a sterile IV catheter was implanted into the right jugular vein and secured with 4-0 Perma-Hand Silk sterile sutures (EthiconEndo-Surgery, Inc). The dorsal portion of the catheter was affixed to an acrylic pedestal embedded with mesh. The dorsal portion/backmount was subcutaneously implanted above the right and left scapulae, and then sterile 4-0 Monoweb sutures were used to stitch the backmount into place. All rats were administered butorphenol (1.0 mg/kg, s.c.) and gentamicin (0.2 ml 1%, i.v.) immediately following the surgery to provide post-operative analgesia and to prevent infection, respectively. Immediately after surgery, animals were monitored in a heat-

regulated warm chamber and returned to the colony room following recovery from anesthesia. All animals survived surgery. The catheters were "flushed" daily with a post-flush solution containing the anti-coagulant heparin (2.5%) and 0.2 ml gentamicin (1%) for an additional 7 days to prevent blood clotting and infections following the surgery. Prior to testing each day, catheters were flushed with a 0.9% saline solution, and following the operant test catheters were treated with the post-flush solution. Cocaine testing began at least 7 days post-surgery.

Cocaine-maintained responding

In brief, the cocaine self-administration procedure was a modified version of Morgan et al., (2006). In our experiments, rats responded for cocaine according to a FR1 schedule of reinforcement, and were switched to daily PR tests with a significantly higher concentration of IV cocaine. The reinforcing efficacy increased over the 14 PR test days, as rats demonstrated a progressive increase in cocaine-maintained responding. We used this procedure to assess potential differences in cocaine-maintained responding between the F344 and HIV-Tg animals.

There were three phases to experiment 3. In phase one, F344 and HIV-Tg rats could respond for cocaine (0.33 mg/kg/inj) according to a FR1 schedule of reinforcement. A 20s time-out, i.e. active levers retracted and the house light was turned off, occurred upon the completion of the ratio requirement. Phase one lasted for five days and each operant test was one hour in duration. In phase 2, animals could respond for cocaine (1.0 mg/kg/inj) according to a PR schedule of reinforcement (Response ratio (rounded to nearest integer) = $[5e^{(\text{injection number} X 0.2)}] - 5$) (Richardson & Roberts, 1996), and this phase lasted for 14 days. The dose of cocaine was 1.0 mg/kg/inj and the operant test was a

maximum of 120 min in duration. The program timed out if 60 min elapsed without the completion of the next response requirement. A 20-s time-out occurred upon completion of the ratio requirement. In the final phase, a PR cocaine dose-response experiment was conducted. The cocaine concentrations were 0.01, 0.03, 0.10, 0.33, and 1.0 mg/kg/inj and they were presented in an ascending manner. A 20s time-out occurred upon the completion of the ratio requirement. Maintenance trials, which made the 0.33 mg/kg/infusion cocaine concentration available on an FR1 schedule, occurred every other day.

Experiment 4: Choice Behavior

Following completion of the cocaine studies, both groups of animals were subject to a choice self-administration protocol. The EC50 for sucrose and cocaine were used as the concentrations for choice behavior. The EC50 for the F344 controls was the training dose (0.33mg/kg/infusion) and due to a lack of differential responding in the HIV-1 Tg animal the training dose was also used as the test dose. Each session began with 4 forced choice (2 nondrug and 2 drug rewards) followed by the choice component. During forced choice, only one active lever was present. The position of the sucrose paired lever (right or left) was balanced between groups. During the choice component, both levers were available simultaneously allowing the animals to freely choose between sucrose and cocaine. During all studies there was an inactive lever located in the center back panel of the chamber.

Experiment 5: Neurochemical measures of the dopamine system [^{3}H] DA uptake in striatum and PFC

Animals were sacrificed within 24 hr following completion of the self-administration choice procedure. The kinetic parameters (V_{max} and K_m) of synaptosomal

[^{3}H]DA uptake were determined using a previously described method (Zhu et al., 2009; Zhu, Green, Bardo, & Dwoskin, 2004). Briefly, brain regions from each rat were homogenized following sacrifice in 20ml of ice-cold sucrose solution (0.32M sucrose and 5mM sodium bicarbonate, pH 7.4) with 16 up-and-down strokes using a Teflon pestle homogenizer (clearance approximately 0.003 inch). The resulting crude synaptosomal preparation was centrifuged at 2,000*g* for 10 min at 4ºC, and the resulting supernatants were centrifuged at 20,000*g* for 15 min at 4ºC. The resulting pellets were resuspended in 4.0ml for PFC and 3.0ml for striatum of ice-cold Krebs-Ringer-HEPES assay buffer (125mM NaCl, 5mM KCl, 1.5mM $MgSO_4$, 1.25 $CaCl_2$, 1.5 KH_2PO_4, 10mM D-glucose, 25mM HEPES, 0.1mM EDTA, 0.1mM pargyline, and 0.1mM L-ascorbic acid, saturated with 95%O_2/5%CO_2, pH 7.4). Half of the striatal synaptosomes from individual rats were used in the [^{3}H]DA uptake assay, and the other half were used for [^{3}H]WIN 35,428 binding assays as described below. The Bradford protein assay (Bradford, 1976) using bovine serum albumin as the protein standard, was used to determine protein concentrations (Bio Rad, USA).

Dopamine is transported by the DAT, the norepinephrine transporter, and the serotonin transporter in the PFC (Moron, Brockington, Wise, Rocha, & Hope, 2002; Williams & Steketee, 2004). In order to isolate the uptake of DA to only DAT within the PFC, kinetic analysis of [^{3}H]DA uptake by the DAT was assessed in the presence of desipramine (1µM) and paroxetine (5nM) to prevent [^{3}H]DA uptake into norepinephrine and serotonin containing nerve terminals (Zhu et al., 2004; Zhu, Apparsundaram, & Dwoskin, 2009).

Synaptosomes containing ~100µg protein/100µl and ~50µg protein/30µl, respectively, were incubated in a metabolic shaker for 10 min at 34ºC and then incubated for 8 min at 34ºC after adding one of 8 [^{3}H]DA concentrations (1nM-1µM, final concentration). Assays were performed in duplicate with a total volume of 500µl. Incubation was terminated by the addition of 3ml ice-cold assay buffer. Samples were immediately filtered through Whatman GF/B glass fiber filters using a Brandel cell harvester (Model M-48' Biochemical Research and Development Laboratories Inc., Gaithersburg, MD). Filters were presoaked with 1mM pyrocatechol in assay buffer and washed 3 times with 3ml ice-cold assay buffer containing 1mM pyrocatechol. Pyrocatechol is a catechol-*O*-methyltransferase (COMT) inhibitor. In the current study, pyrocatechol (1 mM) was included in the assay buffer to prevent the degradation of [^{3}H]DA during the processes of washing and harvesting by COMT (Napolitano, Zurcher, & Da, 1995). Non-specific uptake was determined in duplicate at each [^{3}H]DA concentration by including 10µM nomifensine in the assay buffer. Radioactivity was determined by liquid scintillation spectrometry (Model TRI-CARB 2900TR, Perkin Elmer Instruments, Shelton, CT, USA). GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to determine kinetic parameters (V_{max} and K_m)

Striatal [^{3}H]WIN 35,428 Binding Assay

In order to determine whether [^{3}H]DA uptake into striatal synaptosomes was the result of a direct interaction with the DAT, the maximal number of binding sites (B_{max}) or affinity (K_d) for [^{3}H]WIN 35,428 binding in striatal synaptosomes were examined using a previously described method (Zhu et al., 2009; Zhu, Bardo, Bruntz, Stairs, & Dwoskin, 2007). As described above, half of the striatal synaptosomes from each rat were

centrifuged at 20,000 *g* for 15 min at 4°C and resuspended in 3.0ml ice-cold sodium-phosphate buffer (2.1mM NaH_2PO_4, 7.3mM Na_2HPO_4 $7H_2O$, pH 7.4).

To generate saturation isotherms, striatal synaptosomes containing ~50µg/30µl were added to assay tubes containing one of the eight concentrations of [^{3}H]WIN 35,428 (0.5 to 30 nM, final concentration). Assay tubes were incubated for 2 hr on ice. Nonspecific binding at each concentration of [^{3}H]WIN 35,428 in the presence of cocaine (30µM, final concentration) was subtracted from the total binding in order to calculate specific binding. Rapid filtration (Brandel cell harvester) onto Whatman GF/B glass fiber filters, which were presoaked for 2 hr with assay buffer containing 0.5% polyethylenimine, was used to terminate the assay. Filters were then rinsed 3 times with 3 ml of ice-cold assay buffer. Radioactivity on the filters was determined using liquid scintillation spectrometry (Model TRI-CARB 2900TR, PerkinElmer Instruments, Shelton, CT, USA).

Data Analysis

Data analyses were performed using SPSS version 21 (IBM Corp., Somers, NY), BMDP version 2009 (Statistical Solutions, Saugus, MA), and GraphPad Prism version 5.02 (GraphPad Software, Inc. La Jolla, CA). For experiment 1, a mixed-design analysis of variance (ANOVA) was used to analyze sucrose consumption, with genotype (HIV-1 Tg vs. F344 control) as the between-subjects factor, and sucrose concentration (0-30%, w/v, or day 1-5) as the within-subjects factor. Experiments 2A and 2B were analyzed using an independent samples t-test, using time to meet criterion (60+ reinforcers over 3 days) as the measure of performance. Experiments 2C and 2D were analyzed using a mixed-design ANOVA with genotype (HIV-1 Tg vs. F344 control) as the between-subjects factor and sucrose concentration as the within-subjects factor. Similarly, for

experiments 3A-C, a mixed-design ANOVA was used and genotype was the between-subjects factor and day or concentration was the within-subjects factor. For experiment 4, a 2 (genotype) × 2 (reinforcer type) mixed-design ANOVA was used to analyze the slope of the linear regression lines representing the change in sucrose and cocaine preference over the first 5 days of the choice period (slope values were obtained for each animal for each reinforcer from linear regression analyses on the number of reinforcers obtained over the first 5 days of the choice period). A 2 (genotype) × 2 (reinforcer type) × 5 (day) mixed-design ANOVA was used to analyze number of reinforcers earned and number of active lever press responses. Greenhouse-Geisser corrections were used in cases where Maulchey's tests detected a violation of sphericity.

For experiments 5 and 6, which are the DA uptake and binding experiments, the data are presented as mean values ±S.E.M.; *n* represents the number of independent experiments for each treatment group. Kinetic parameters of [^{3}H]DA uptake (V_{max} and K_m) and [^{3}H]WIN 35,428 binding (B_{max} and K_d) were determined from saturation curves by nonlinear regression analysis using a one-site model with variable slope. To determine the differences in the kinetic parameters, separate two-way ANOVAs were used to evaluate any differences between genotype (HIV-1 Tg vs. F344 control) and treatment (cocaine or sucrose) between-subject factors. Simple pairwise comparisons were used to evaluate planned-comparisons. Log transformed values of K_m or K_d were utilized for statistical comparisons. Differences were considered significant at $p \leq 0.05$.

Stepwise regression was used to detect any significant behavioral predictors of neurochemical changes observed in the DA uptake and binding experiments. Significant

models were further explored using multiple linear regression analyses. Models were considered significant if $p \leq 0.05$.

An exploratory discriminant function analysis was employed to determine which neurochemical measures best differentiated group performance, and the extent to which the observed differences in measures correctly identified animals in regard to their group membership (HIV-1 Tg vs. F344 control).

Results

Motivational alterations in HIV-1+ individuals are associated with decreased performance on tasks known to involve the frontal-subcortical circuitry (Cole et al., 2007), and associated with decreased volume of the nucleus accumbens (Paul et al., 2005b). Apathy is not generally addressed in animal models; however, the concept of motivation, or how willing an animal is to "work" for reinforcement, is frequently tested in animals using operant conditioning. In the current experiments, operant schedules of reinforcement were used to assess potential differences in sensitivity to the reinforcer (i.e., FR;Yokel & Pickens, 1973; Arnold & Roberts, 1997) and changes in reinforcement efficacy (i.e., PR;Morgan, Liu, & Roberts, 2006) between control and transgenic rats that constitutively express multiple proteins associated with the HIV-1 virus.

The HIV-1 Tg rat does not exhibit altered sucrose taste preference

We first examined the ability of HIV-1 Tg rats to detect sucrose and whether any taste preference might bias subsequent testing of the HIV-1 Tg rats using sucrose as a reinforcer (Figure 5.1A). A 2 × 5 × 5 mixed-design ANOVA revealed a significant main effect of concentration [$F(4, 108)=55.03$, $p_{GG} \leq 0.001$], and a significant day × concentration interaction [$F(16, 432)=3.05$, $p_{GG} \leq 0.05$]. There was no significant main

effect of genotype [F(1,27)<1.0]. As illustrated (Figure 5.1C), the orthogonal components of the significant two-way interaction indicated fluid consumption was a robust linear function of sucrose concentration from 0 - 30% w/v [F(1,27)=81.81, $p \leq 0.001$]. Sucrose preference was not altered in the HIV-1 Tg rats, relative to F344 controls.

Critically, bottle position was also evaluated in order to assess any potential confound with animal side bias or preference, as illustrated in the Latin-Square design (Figure 5.1B). A 2 × 5 × 5 mixed-design ANOVA of bottle position revealed no significant main effect of bottle position, day, or day × position interaction (Figure 5.1D) indicating that the sucrose taste preference results were not altered by any positional or side bias of the animals.

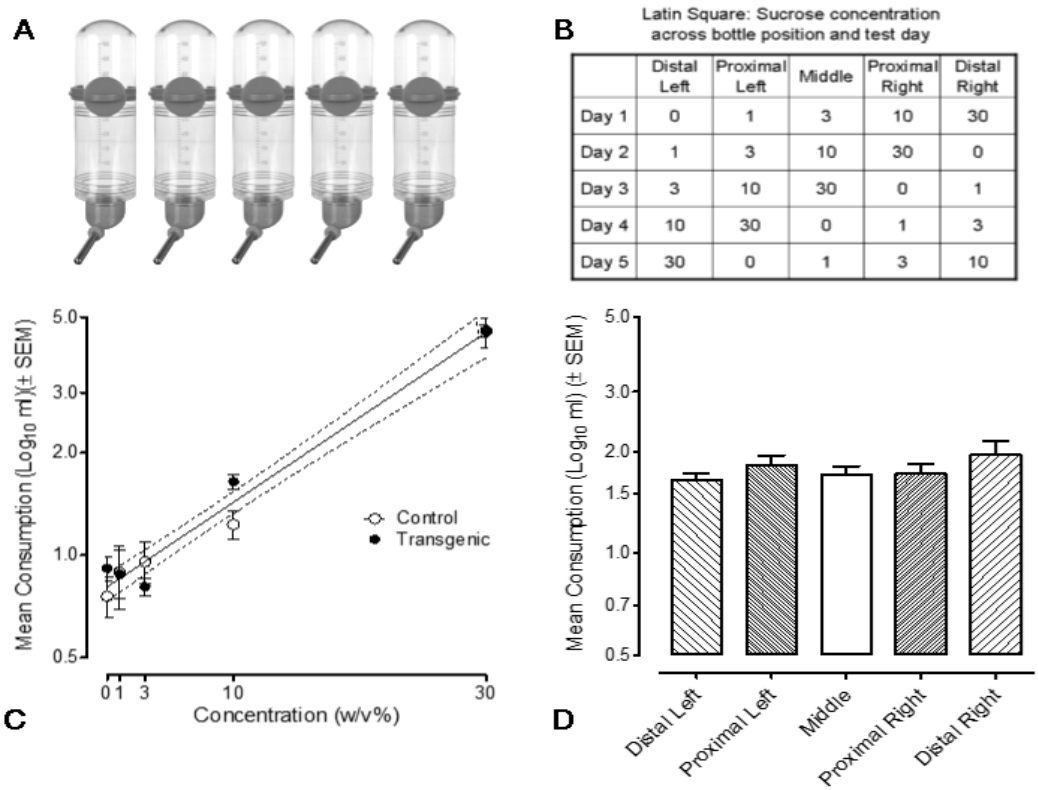

Figure 5.1 The HIV-1 Tg rat does not exhibit altered sucrose preference. Prior to the evaluation assessing the effects of long-term HIV-1 protein exposure on motivated behavior using sucrose, an assessment of sucrose preference was conducted. **A**. Animals had free access for 1 hour to five bottles filled with 0, 1, 3, 10, or 30% (w/v) sucrose

solutions; bottles were weighed and menisci read to the nearest ml before and after each test. **B.** To control for a position bias, bottle order was randomized for each rat on the first day of testing and bottles were rotated on each subsequent day using a Latin square procedure so that each concentration was in each location once. **C.** Mean (±SEM) consumption (ml) of each solution over the 5 testing days illustrated that regardless of genotype, fluid consumption was a robust linear function of sucrose concentration [$F(1,27)=81.81$, $p\leq0.001$; ±95%CI]**.** **D.** Mean (±SEM) fluid consumption from each bottle position across days revealed there was no confound with any positional or side bias of the animals, i.e., there was no significant effect of bottle position, day, or a day x position interaction.

HIV-1 Tg rats successfully acquire sucrose-maintained responding under either restricted or non-restricted conditions

Given that there were no alterations in sucrose preference, operant procedures were used to determine sensitivity to sucrose reinforcement (Figure 5.2A). The proportion of animals (HIV-1 Tg or F344 controls) meeting criterion across days under conditions of water restriction, as illustrated (Figure 5.2B**)** showed there was no overall effect of the HIV-1 transgene on the number of animals that were able to acquire the task; at the end of 68 days, 100% of the F344 controls and 93% of the HIV-1 Tg rats met criterion (15/15 vs. 13/14, $z=1.05$, $p\leq0.29$). However, also illustrated is the observation that the HIV-1 Tg animals took longer (a mean of 20 additional days) to reach criterion (60+ reinforcers for 3 consecutive days) relative to F344 controls. A linear regression on the proportion data revealed that the results for both genotypes were a significantly linear fit (r^2= 0.9219 and r^2= 0.9427 for F344 and HIV-1 Tg animals, respectively) and further confirmed that the slopes of the lines were significantly different from one another [$F(1,99)=177.08$ $p\leq0.001$].

Once animals met criterion for 3 consecutive days under water restriction, they were allowed *ad libitum* access to water in their home cages and were tested on an FR1 schedule of reinforcement. The proportion of animals (HIV-1 Tg or F344 controls) meeting criterion across days without extrinsic motivation (no water restriction), as

illustrated (Figure 5.2C**)**, showed there was no overall effect of the HIV-1 transgene on the number of animals that were able to acquire the task; at the end of 68 days, 80% of the F344 controls and 54% of the HIV-1 Tg rats met criterion [12/15 vs. 7/13, z=1.48, $p \leq 0.14$]. Again, also illustrated is the observation that the HIV-1 Tg animals took longer (a mean of 20 additional days) to reach criterion (60+ reinforcers for 3 consecutive days), relative to F344 controls. Specifically, regression on the proportion of animals meeting criterion across days revealed robust linear functions (r^2=0.95 and r^2=0.89 for F344 and HIV-1 Tg animals, respectively) and confirmed that the slopes of the lines were significantly different from one another [$F(1,74)=193.30$, $p \leq 0.001$].

Thus, HIV-1 Tg rats successfully acquired the self-administration task, under either restricted or even non-restricted conditions, albeit the rate at which the groups acquired the task was significantly slower as a function of expression of the HIV-1 transgene.

Response vigor for, but not sensitivity to, or reinforcing efficacy of, sucrose reinforcement was diminished in the HIV-1 Tg rat across multiple sucrose concentrations

Next, a FR schedule of reinforcement was used to determine if there were any detectable differences in sensitivity to sucrose as a reinforcer across multiple concentrations of sucrose (i.e., FR;Yokel & Pickens, 1973; Arnold & Roberts, 1997). A 2 x 6 repeated-measures ANOVA on total earned reinforcers revealed a main effect of genotype [$F(1,27)=10.7$ ($p \leq .003$)], a main effect of concentration [$F(5, 135)=65.9$, $p_{GG} \leq 0.001$], and more importantly, a genotype x concentration interaction [$F(5,135)=5.2$, $p_{GG} \leq 0.006$]. More specifically, as illustrated (Figure 5.2D), a sigmoidal curve fit provided a robust characterization of the dose-response function for both HIV-1 Tg and control animals (r^2=0.99 for both the HIV-1 Tg and F344 control groups). The curve was

shifted significantly downward [$F(4,2)=1194.0$, $p\leq 0.001$], however, there was no significant shift in the EC_{50}; i.e., although there was clear evidence of a decrease in overall response vigor as suggested by the overall decrease in responding, there was no compelling evidence for any change in sensitivity to sucrose as a reinforcer in the HIV-1 Tg animals.

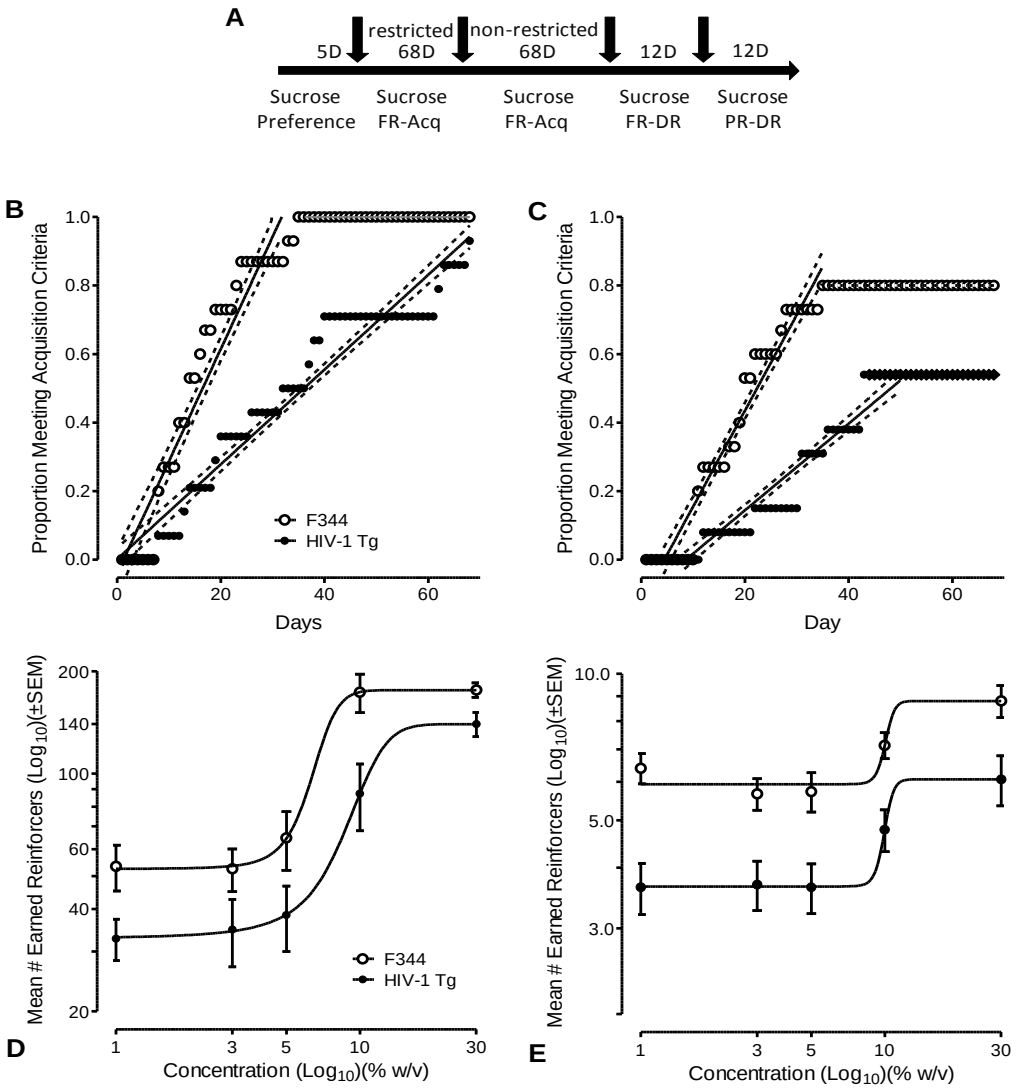

Figure 5.2 Response vigor for, but not sensitivity to, or reinforcing efficacy of, sucrose reinforcement was diminished in the HIV-1 Tg rat across multiple sucrose concentrations. **A.** Given that there were no alterations in sucrose preference, operant procedures were used to determine sensitivity to sucrose reinforcement. **B.** HIV-1 Tg rats successfully acquired (criterion of 60+ reinforcers for 3 consecutive days) sucrose-

maintained responding under conditions of 12-15 hr of water restriction. No overall effect of the HIV-1 transgene on the number of animals that were able to acquire the task was detected after 68 days (15/15 vs. 13/14, F344 controls and the HIV-1 Tg rats, respectively), however, the HIV-1 Tg animals took longer to reach criterion relative to F344 controls (slopes of the lines were significantly different from one another [$F(1,99)=177.08$ $p\leq0.001$]). **C.** HIV-1 Tg rats successfully acquired (criterion as above) sucrose-maintained responding without extrinsic motivation (no water restriction). No overall effect of the HIV-1 transgene on the number of animals that were able to acquire the task was found at the end of 68 days (12/15 vs. 7/13, F344 controls and the HIV-1 Tg rats, respectively]. Again, however, the HIV-1 Tg animals took longer to reach criterion, relative to F344 controls (slopes of the lines were significantly different from one another [$F(1,74)=193.30$, $p\leq0.001$]). **D.** A robust characterization of the dose-response function (1-30% w/v sucrose) under the FR schedule of reinforcement was provided for both HIV-1 Tg and control animals by a sigmoidal curve fit ($r^2=0.99$ for both the HIV-1 Tg and F344 control groups). The curve was shifted significantly downward [$F(4,2)=1194.0$, $p\leq0.001$], however, there was no significant shift in the EC_{50}, providing no compelling evidence for any change in sensitivity to sucrose as a reinforcer in the HIV-1 Tg animals. **E.** Similarly, a robust characterization of the dose-response function (1-30% w/v sucrose) under the PR schedule of reinforcement was provided for both HIV-1 Tg and control animals by a sigmoidal curve fit ($r^2=0.99$ and $r^2=0.95$ for HIV-1 Tg and F344 controls, respectively). ($r^2=0.99$ and $r^2=0.95$ for HIV-1 Tg and F344 controls, respectively). The curve was significantly shifted downward [$F(3,4)=57.42$, $p\leq0.001$], however, again, there was no significant shift in the EC_{50}, providing no compelling evidence for any change in efficacy of sucrose as a reinforcer in the HIV-1 Tg animals.

A PR schedule of reinforcement was then used to assess potential differences in reinforcement efficacy of sucrose across a range of concentrations (i.e., PR;Morgan et al., 2006). A 2x6 repeated-measures ANOVA on total earned reinforcers revealed a main effect of genotype [$F(1,27)=18.52$, $p\leq0.001$], and a main effect of concentration [$F(5,135)=9.071$, $p_{GG}\leq0.001$] but no significant concentration x genotype interaction. A sigmoidal dose-response analysis, as illustrated (Figure 5.2E), again confirmed a robust characterization of the dose-response function for both HIV-1 Tg and control animals ($r^2=0.99$ and $r^2=0.95$ for HIV-1 Tg and F344 controls, respectively). The curve was significantly shifted downward [$F(3,4)=57.42$, $p\leq0.001$], however, again, there was no significant shift in the EC_{50}; i.e., the clear evidence of a decrease in response vigor as

indexed by the decreased overall responding was not accompanied by any evidence for an alteration in efficacy of sucrose as a reinforcer in the HIV-1 Tg animals.

Sensitivity to, and reinforcing efficacy of, cocaine is diminished in the HIV-1 Tg rats relative to F344 control rats

An FR1 schedule of reinforcement was used to train the animals to self-administer cocaine as well as to assess any potential differences in sensitivity to cocaine as a reinforcer with daily access to a moderate dose of cocaine (0.33mg/kg/infusion). As illustrated (Figure 5.3B), a 2 × 5 repeated-measures ANOVA revealed a significant effect of genotype with HIV-1 Tg animals receiving fewer infusions that F344 controls [$F(1,19)=4.8$, $p\leq0.05$]. There was no significant effect of day nor was there any significant orthogonal component to characterize a trend across days, indicating that the animals neither escalated nor regressed in their drug intake. However, the data were significantly greater than zero, suggesting that this dose of cocaine was not aversive to these animals.

Following FR1 training, animals underwent 14 consecutive days of PR responding for a dose of cocaine three times that of the training dose (1.0mg/kg/infusion). As illustrated (Figure 5.3C), a 2 × 14 mixed-design ANOVA on number of cocaine infusions earned indicated a significant effect of genotype [$F(1,13)=45.3$, $p\leq0.001$], and a significant main effect of day [$F(13, 169)=2.9$, $p_{GG}\leq0.05$]. Orthogonal decomposition of the day effect confirmed a prominent linear increase consistent with an escalation of cocaine intake across days [$F(1,13)=10.0$, $p\leq0.01$]. Regression analyses substantiated a prominent linear fit for both genotypes ($r^2= 0.67$ and $r^2= 0.66$ for F344 and HIV-1 Tg animals, respectively) and further confirmed that the slopes of the lines were significantly different from one another [$F(1,24)=6.3$ $p\leq0.02$]. Thus, differential escalation to cocaine

was observed with the HIV-1 Tg animals showing a significantly slower rate relative to F344 controls.

A PR schedule with 5 different concentrations of cocaine was subsequently employed to assess how willing the animals were to work for cocaine with the establishment of their respective dose-response functions. As illustrated (Figure 5.3D), a 2 × 5 mixed-design ANOVA on infusions earned found an overall significant effect of genotype with HIV-1 Tg animals earning significantly fewer infusions of cocaine relative to F344 controls [$F(1,13)=6.4$, $p\leq0.025$]. Regression analyses demonstrated a linear log-log fit for both genotypes ($r^2= 0.52$ and $r^2= 0.33$ for F344 and HIV-1 Tg animals, respectively) and further confirmed that the slopes of the lines were significantly different from one another [$F(2,6)=67.5$, $p\leq0.001$]. Similarly, as illustrated (Figure 5.3E), a 2 x 5 mixed-design ANOVA on response break point found an overall effect of genotype with HIV-1 Tg animals achieving a significantly lower break point [$F(1,12)=8.8$, $p\leq0.012$]. Regression analyses confirmed a linear log-log fit for both genotypes ($r^2= 0.81$ and $r^2= 0.25$ for F344 and HIV-1 Tg animals, respectively) and demonstrated that the slopes of the lines were significantly different from one another [$F(2,6)=30.0$, $p\leq0.001$]. Thus, sensitivity to, and reinforcing efficacy of, cocaine was diminished in the HIV-1 Tg rats relative to F344 control rats.

Choice between sucrose and cocaine indicated change in preference

Since the animals had a history of responding for sucrose as well as cocaine, and the animals were also trained to preclude the development of a response side bias, the choice behavior for sucrose vs. cocaine was subsequently assessed (Figure 5.4A). Regression analyses on the number of reinforcers earned across days, collapsed across genotype (Figure 5.4B), substantiated a prominent decrease in sucrose reinforcers earned (linear,

r^2= 0.85) vs. a prominent increase in cocaine infusions earned (single phase association, r^2= 0.88). The initial choice for sucrose over cocaine by a greater than 2:1 ratio shifted across the 7-day period to a greater than 2:1 ratio for cocaine over sucrose. The rate of change was -3.4±0.63 for sucrose vs. 1.1±0.35 for cocaine, significantly different from each other [F(1,10)=38.7, $p \leq 0.001$] and from zero ([F(1,5)=28.5, $p \leq 0.005$ and F(1,5)=10.3, $p \leq 0.025$], respectively). With the replacement of cocaine with saline on day 8, the drug extinction day, the choice shifted to a return for sucrose over cocaine by an approximate 2:1 ratio.

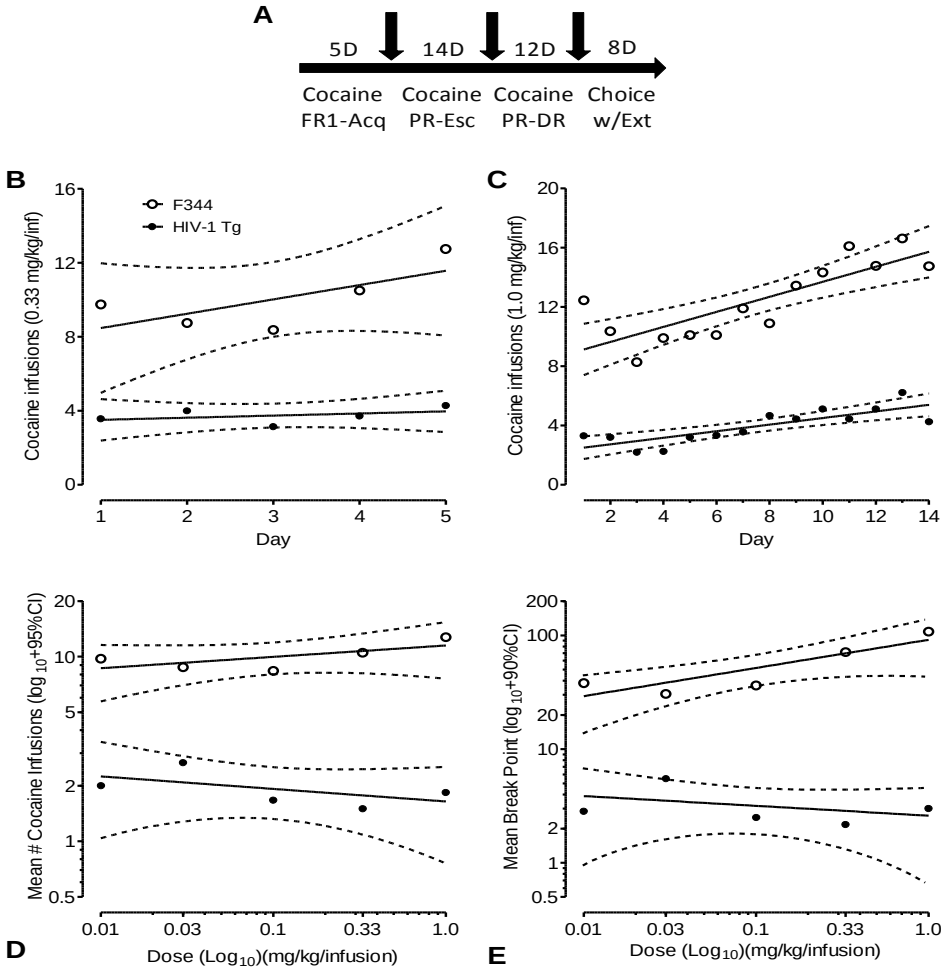

Figure 5.3 Sensitivity to, and reinforcing efficacy of, cocaine was diminished in the HIV-1 Tg rats relative to F344 control rats. **A.** Cocaine self-administration (acquisition,

escalation, and PR dose-response) was employed to assess potential differences in cocaine-maintained responding between the F344 and HIV-Tg animals. **B.** The mean number of infusions (±95% CI) earned during the 5 day cocaine acquisition period demonstrated that both HIV-1 Tg and F344 animals maintained a stable response during the acquisition period, however, the HIV-1 Tg animals received significantly fewer infusions than F344 controls [$F(1,19)=4.8$, $p\leq0.05$]. **C.** The mean number of infusions (±95% CI) earned across the 14-day PR sessions revealed robust effects of genotype [$F(1,13)=45.3$, $p\leq0.001$] and day [$F(13,169)=2.9$, $p_{GG}\leq0.05$], which was functionally linear [$F(1,13)=10$, $p\leq0.01$], indicative of an escalation of cocaine intake across sessions. Regression analyses substantiated a prominent linear fit for both genotypes (r^2= 0.67 and r^2= 0.66 for F344 and HIV-1 Tg animals, respectively) and further confirmed that the slopes of the lines were significantly different from one another [$F(1,24)=6.3$ $p\leq0.02$]. Escalation to cocaine was significantly slower for the HIV-1 Tg animals rate relative to F344 controls. **D.** Number of infusions (±95% CI) earned and ***E*.** last ratio completed (break point) during cocaine dose-response sessions under the PR schedule of reinforcement demonstrated that the F344 animals received more cocaine infusions as dose increased [$F(1,13)=18.77$, $p\leq0.02$], whereas the responding of the HIV-1 Tg animals did not vary as a function of increased cocaine dose. The slopes of the linear log-log fits for number of infusions earned (r^2= 0.52 and r^2= 0.33 for F344 and HIV-1 Tg animals, respectively) were significantly different from one another [$F(2,6)=67.5$, $p\leq0.001$]. Similarly, the slopes of the linear log-log fits for break point (r^2= 0.81 and r^2= 0.25 for F344 and HIV-1 Tg animals, respectively) were significantly different from one another [$F(2,6)=30.0$, $p\leq0.001$]. Thus, sensitivity to, and reinforcing efficacy of, cocaine was diminished in the HIV-1 Tg rats relative to F344 control rats.

The choice behavior of the F344 control group (Figure 5.4C) and HIV-1 Tg animals (Figure 5.4D) was subsequently examined. The rate of change in choice behavior, captured by a 2 × 2 × 5 repeated measures ANOVA on the number of reinforcers received, revealed a significant genotype × reinforcer type × day interaction [$F(4,36) = 2.95$, $p\leq0.05$]. For the F344 control group, regression analyses confirmed a prominent decrease in sucrose reinforcers earned (single phase decay, r^2= 0.96) vs. a prominent increase in cocaine infusions earned (single phase association, r^2= 0.78). The initial choice for sucrose over cocaine by a greater than 2:1 ratio shifted within 5 days to a greater than 2:1 ratio for cocaine over sucrose. The rate of change across the 5-day period before reaching their response plateau was -8.8 ±0.99 for sucrose vs. 3.5±1.08 for

cocaine, significantly different from each other [F(2,6)=39.8, $p \leq 0.001$] and from zero ([F(1,3)=79.5, $p \leq 0.005$ and F(1,3)=10.8, $p \leq 0.05$], respectively). With the replacement of cocaine with saline on day 8, the drug extinction day, the choice shifted to a return for sucrose over cocaine by an approximate 2:1 ratio. The F344 control group displayed a significantly greater number of active lever presses for sucrose on day 8 than they did on day 7, the last day of choice, (t(5)= -2.8, $p \leq 0.05$), and had returned to a level that was not significantly different from the number of active lever presses for sucrose on the first day of the choice period.

For the HIV-1 Tg group, regression analyses confirmed a prominent decrease in sucrose reinforcers earned (linear, r^2= 0.72) vs. a nominal decrease in cocaine infusions earned (linear, r^2= 0.008). The initial choice for sucrose over cocaine by a greater than 2:1 ratio shifted across the 7-day period to no choice preference for cocaine vs. sucrose. The rate of change across the 7-day period was -2.3 ±0.65 for sucrose vs. -0.07±0.34 for cocaine, significantly different from each other [F(2,10)=6.9, $p \leq 0.015$]. The rate decrease in sucrose reinforcers earned was significantly different from zero ([F(1,5)=12.7, $p \leq 0.025$], not unlike that noted in the choice behavior of the F344 control animals. However, no rate change was discernible for cocaine infusions earned across the 7-day period (not significantly different from zero, F(1,5)<1.0]), despite the fact that the HIV-1 Tg animals nevertheless chose cocaine infusions at a mean level greater than zero [F(1,6)=74.2, $p \leq 0.001$]. With the replacement of cocaine with saline on day 8, the drug extinction day, no differential choice was observed for the sucrose vs. cocaine response levers. Collectively, choice behavior was significantly disrupted by the HIV-1 transgene;

i.e., although responding of the HIV-1 Tg animals was sensitive to the reinforcing properties of cocaine, it was not driven by any increase in choice behavior for cocaine.

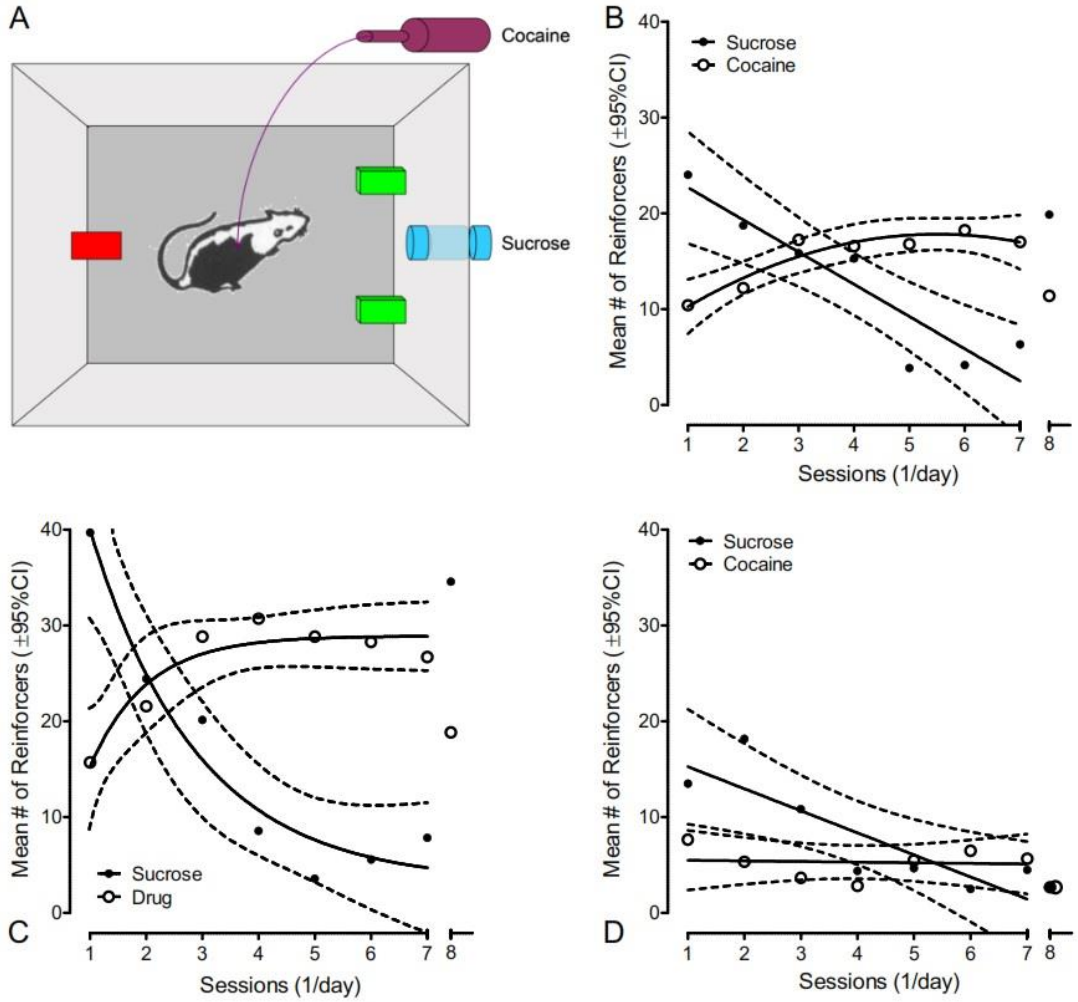

Figure 5.4 HIV-1 transgene disrupts choice behavior. **A.** With a history of responding for sucrose as well as cocaine as well as explicit training to preclude the development of a response side bias, **B.** the choice behavior of the animals across days, collapsed across genotype, displayed a prominent decrease in sucrose reinforcers earned (linear, r^2= 0.85) vs. a prominent increase in cocaine infusions earned (single phase association, r^2= 0.88). The initial choice for sucrose over cocaine by a greater than 2:1 ratio shifted across the 7-day period to a greater than 2:1 ratio for cocaine over sucrose; with the replacement of cocaine with saline on day 8, the drug extinction day, the choice shifted to a return for sucrose over cocaine by an approximate 2:1 ratio. Given the significant genotype × reinforcer type × day interaction [$F(4,36) = 2.95$, $p \leq 0.05$], the choice behavior of the F344 control group (**C**) and HIV-1 Tg animals (**D**) is displayed. **C.** For the F344 control

group, their choice behavior displayed a prominent decrease in sucrose reinforcers earned (single phase decay, r^2= 0.96) vs. a prominent increase in cocaine infusions earned (single phase association, r^2= 0.78). The initial choice for sucrose over cocaine by a greater than 2:1 ratio shifted within 5 days to a greater than 2:1 ratio for cocaine over sucrose. With the replacement of cocaine with saline on day 8, the drug extinction day, the F344 control group displayed a significantly greater number of active lever presses for sucrose on day 8 than they did on day 7, the last day of choice, ($t(5)= -2.8$, $p\leq0.05$), and had returned to a level that was not significantly different from the number of active lever presses for sucrose on the first day of the choice period. **D.** For the HIV-1 Tg group, their choice behavior also displayed a a prominent decrease in sucrose reinforcers earned (linear, r^2= 0.72) vs. only a nominal decrease in cocaine infusions earned (linear, r^2= 0.008). The initial choice for sucrose over cocaine by a greater than 2:1 ratio shifted across the 7-day period to no choice preference for cocaine vs. sucrose. No rate change was discernible for cocaine infusions earned across the 7-day period (not significantly different from zero, $F(1,5)<1.0$]), despite the fact that the HIV-1 Tg animals nevertheless chose cocaine infusions at a mean level greater than zero [$F(1,6)=74.2$, $p\leq0.001$]. With the replacement of cocaine with saline on day 8, the drug extinction day, no differential choice was observed for the sucrose vs. cocaine response levers. Thus, choice behavior was significantly disrupted by the HIV-1 transgene; i.e., although responding of the HIV-1 Tg animals was sensitive to the reinforcing properties of cocaine, it was not driven by any increase in choice behavior for cocaine.

Dopamine Transporter in the HIV-1 Tg rat

To determine the relationship between brain neurochemistry changes in the DAT and behavior, animals were sacrificed within 24 hr of the last cocaine self-administration session. The brains were rapidly dissected and used immediately for neurochemical analyses. These and other relevant neurochemical findings regarding dopamine terminal regulation are summarized in Figures 5.5 D-G.

V_{max} increases in F344 controls, but not in HIV-1 Tg animals, following cocaine self-administration

A 2 × 2 ANOVA was used to analyze the effects of cocaine self-administration and genotype on V_{max} and K_m in the striatum. There was a significant genotype by treatment interaction [$F(1,20)=7.8$, $p\leq0.01$] with V_{max} as the dependent variable. As illustrated (Figure 5.5A), F344 control rats with a history of cocaine self-administration displayed a significantly increased V_{max}, compared to F344 sucrose rats ($p\leq0.05$).

Conversely, cocaine self-administration did not significantly affect V_{max} in HIV-1 transgenic rats compared to HIV-1 Tg sucrose animals. F344 control animals with a history of cocaine self-administration had significantly higher V_{max} rates than HIV-1 Tg animals with a history of cocaine self-administration ($p \leq 0.05$). There were no significant effects of cocaine self-administration or genotype on K_m in the striatum.

In the prefrontal cortex, a similar pattern emerged. There was a significant genotype by treatment interaction [$F(1,20)=6.1$, $p \leq 0.02$] with V_{max} as the dependent variable. HIV-1 Tg animals displayed a significantly reduced V_{max} following cocaine self-administration when compared with cocaine naïve HIV-1 Tg rats ($p \leq 0.05$), while the F344 control animals did not have an altered V_{max} following cocaine self-administration. HIV-1 Tg rats with a history of cocaine self-administration displayed a significantly decreased V_{max} compared to F344 control rats with a history of cocaine self-administration, $p \leq 0.05$. There were no significant effects of cocaine self-administration or genotype on K_m in the PFC.

B_{max} is significantly decreased in HIV-1 Tg animals and increases following cocaine self-administration

Analysis of maximum binding (B_{max}) using a two-way ANOVA revealed a significant main effect of genotype [$F(1,18)=4.58$, $p \leq 0.05$]. HIV-1 Tg sucrose rats had a significantly lower maximum [^{3}H]WIN 35,428 binding, relative to F344 sucrose animals. Additionally, a significant genotype × treatment (cocaine vs. sucrose) interaction was also detected [$F(1,18)=4.15$, $p \leq 0.05$]. As illustrated (Figure 5.5B), F344 sucrose control rats had a significantly higher B_{max} compared to HIV-1 Tg cocaine naïve rats ($p \leq 0.05$). B_{max} in HIV-1 Tg animals with a history of cocaine self-administration, was not significantly different from F344 animals which had also self-administered cocaine.

Decreased DA turnover rate in the striatum of the HIV-1 Tg rat following cocaine self-administration

DA turnover rate was determined by dividing striatal V_{max} by striatal B_{max}. Using the resulting turnover values, a two-way ANOVA was used to determine effects of cocaine self-administration on DA turnover in these animals. A significant genotype × treatment interaction was noted [$F(1,18)=6.74$, $p\leq0.02$], albeit no main effects of genotype or treatment were found. As illustrated (Figure 5.5C), F344 animals had a significantly increased DA turnover rate following cocaine self-administration ($p\leq0.05$); in contrast, HIV-1 Tg animals showed a significant decrease in DA turnover following cocaine self-administration ($p\leq0.05$).

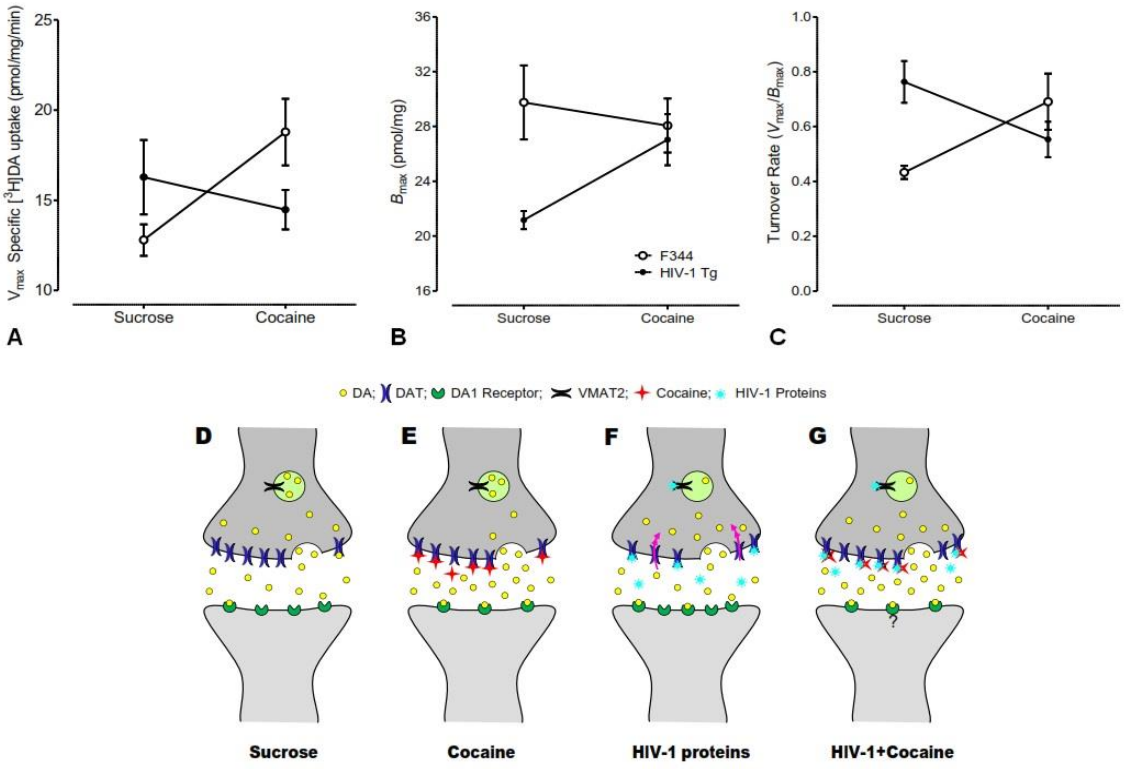

Figure 5.5 Dopamine transporter dysregulation in HIV-1 Tg animals and interactions with cocaine self-administration. **A.** [^{3}H]DA uptake in striatal synaptosomes from sucrose and cocaine self-administering animals (Mean ± SEM). A significant genotype by treatment interaction was detected $F(1,20)=7.79$, $p\leq0.01$. Cocaine self-administration in F344 animals produced a significant increase in V_{max}, $p\leq0.05$, compared to sucrose F344 controls. There was no significant effect of cocaine self-administration on V_{max} in

the HIV-1 Tg rat, relative to sucrose; however, cocaine self-administration in HIV-1 Tg animals produced a significantly lower V_{max} , in comparison to cocaine self-administration in F344 animals, $p \leq 0.05$. There were no significant effects of genotype, treatment, or a significant interaction, on K_m in the striatum. All experiments were performed in duplicate, tissue from each animal was considered an individual experiment. **B**. [^{3}H]WIN 35,428 binding to DAT in striatal synaptosomes (Mean ± SEM). HIV-1 Tg sucrose rats had a significantly lower B_{max} in comparison to F344 sucrose animals, $F(1,18)=4.58$, $p \leq 0.05$. Cocaine self-administration had a significant interaction with genotype $F(1,18)=4.15$, $p \leq 0.05$; cocaine self-administering HIV-1 Tg rats increased B_{max} to those of cocaine self-administering F344 animals. There was no significant effect of genotype or drug treatment on K_d in striatal synaptosomes. **C.** Dopamine turnover rate was determined via V_{max}/B_{max}. A significant genotype by treatment interaction was found $F(1,18)=6.74$, $p \leq 0.02$. Cocaine self-administration produced a significantly increased turnover rate in F344 animals $p \leq 0.05$ and, conversely, a significantly decreased turnover rate in HIV-1 Tg animals $p \leq 0.05$.**D,E**. Illustration of a dopamine terminal following sucrose (**D**) and cocaine (**E**) self-administration in F344 animals. Cocaine self-administration leads to increased rates of dopamine uptake (Ramamoorthy, Samuvel, Balasubramaniam, See, & Jayanthi, 2010); increased dopamine turnover (Goeders & Smith, 1993); increased phasic concentrations of DA (Pettit & Justice, Jr., 1991); and decreased post-synaptic D1 receptors (Graziella de Montis et al., 1998), relative to sucrose animals. **F**. Illustration of dopamine terminal in HIV-1 Tg animals. HIV-1 proteins (tat, gp120) modulate DAT function (Zhu et al., 2009; Midde et al., 2013; Wallace et al., 2006) and VMAT2 (Midde et al., 2012); more efficient DAT function/higher turnover rate and no increase in DA dopamine concentration (Ferris et al., 2010). Increased postsynaptic D1 receptors in HIV-1 (Silvers et al. 2007; Aksenov et al., 2006).**G**. Illustration of dopamine terminal following cocaine self-administration in HIV-1 Tg animals. Cocaine and HIV-1 proteins both bind to the DAT protein (Zhu et al., 2011); HIV-1 proteins bind to VMAT2 (Midde et al., 2012); DAT funding is less efficient, resulting in lower turnover rate of dopamine. Dopamine concentrations increase in the presence of both HIV-1 proteins and cocaine, producing chronic hyperdopaminergic tone (Ferris et al., 2010). Status of D1 postsynaptic receptors is unknown.

Behavior predicting neurochemical outcomes: Cocaine and sucrose

Cocaine. The cocaine FR acquisition period was determined to significantly predict V_{max} in the PFC (data from each day were considered to be independent predictors, [$F(5,8)=12.374$, $p \leq .005$]). The adjusted r^2 indicated that 81.4% of the variance in PFC V_{max} was associated with number of infusions received on days 1-5 of cocaine acquisition. Cocaine self-administration produced a significant decrease in [^{3}H]DA V_{max} in the HIV-1 Tg rat PFC in comparison to both HIV-1 Tg drug-naïve animals and F344 cocaine self-administration animals. In contrast, cocaine self-administration did not

significantly affect [^{3}H]DA V_{max} in the PFC of F344 animals, which is similar to previous findings (Grimm, Shaham, & Hope, 2002; Miguens et al., 2008; Ben-Shahar, Moscarello, & Ettenberg, 2006; Williams & Steketee, 2004). Interestingly, the number of cocaine infusions received during cocaine acquisition significantly predicted the neurochemical changes in the prefrontal cortex, suggesting that dopamine in the prefrontal cortex plays a significant role in establishing cocaine reinforcement in the HIV-1 Tg rat.

The cocaine PR escalation period was also determined to significantly predict V_{max} in the PFC using multiple linear regression (data from each day – day 1 to day 14 -- were considered to be independent predictors, [$F(7,4)=20.57$, $p\leq.01$]). The adjusted r^2 indicated that 92.6% of the variance in PFC V_{max} was associated with the number of lever presses across the 14-day period of cocaine escalation. The number of active lever presses during the cocaine PR dose-response experiment (data from each dose were considered independent predictors) was determined to significantly predict both V_{max} in the striatum [$F(5,6)=15.77$, $p\leq0.005$] and DA turnover [$F(5,6)=17.07$, $p\leq0.005$]. The adjusted r^2 values indicated that 87% of the variance in STR V_{max} and 88% of the variance in DA turnover were associated with the number of active lever presses during the cocaine PR dose-response experiment. The number of infusions during the cocaine PR dose-response experiment (data from each dose were considered independent predictors) was determined to significantly predict B_{max} in the striatum [$F(5,6)=6.4$, $p\leq0.05$]. The adjusted r^2 indicated that 71.3% of the variance in STR B_{max} was associated with the number of infusions received during the cocaine PR dose-response experiment. Thus, those neurochemical measures that were significantly predicted by behavioral

measures were PFC V_{max}, STR V_{max}, STR B_{max}, and DA turnover in multiple regression analyses.

Sucrose. FR responding for sucrose did not predict neurochemical outcomes. The number of active lever presses during the sucrose PR dose-response experiment (data from each concentration were considered independent predictors) was determined to significantly predict both V_{max} in the striatum [$F(6,15)=2.95$, $p\leq0.05$] and DA turnover in the striatum [$F(6,15)=3.134$, $p\leq0.05$] using multiple linear regression. The adjusted r^2 values indicated that 35.7% of the variance in STR V_{max} and 37.9% of the variance in DA turnover were associated with the number of active lever presses during the sucrose PR experiment. The slope of the linear regression lines representing the change in sucrose preference over the first 5 days of the choice period significantly predicted B_{max} in the striatum in the control group [$F(1,4)=8.3$, $p\leq0.05$], but not in the HIV-1 Tg group. The adjusted r^2 indicated that 59.6% of the variance in STR B_{max} was associated with the change in sucrose preference over the 5-day period. Relative to the number of significant behavioral:neurochemical correlations found for cocaine reinforcement, far fewer behavioral alterations for sucrose reinforcement predicted alterations in the dopamine neurochemistry.

Discriminant Function Analysis

A discriminant function analysis was conducted to further explore the effects of expression of the HIV-1 transgene (Figure 5.6), by assessing the ability of various neurochemical measures to correctly identify group membership of individual animals (HIV-1 Tg vs. control). The predictor variables that were selected for the analysis (PFC V_{max}, STR V_{max}, STR B_{max}, and DA turnover) were those neurochemical measures that were significantly predicted by behavioral measures in multiple regression analyses (as

noted above). The discriminant function revealed a significant association between groups and all predictors, accounting for 67.2% of between group variability (canonical correlation of 0.82) and correctly classified the animals' group membership with 92.9% accuracy (100% of HIV-1 Tg animals, and 85.7% of controls) (Wilks' $\lambda = 0.328$, $p \leq 0.05$).

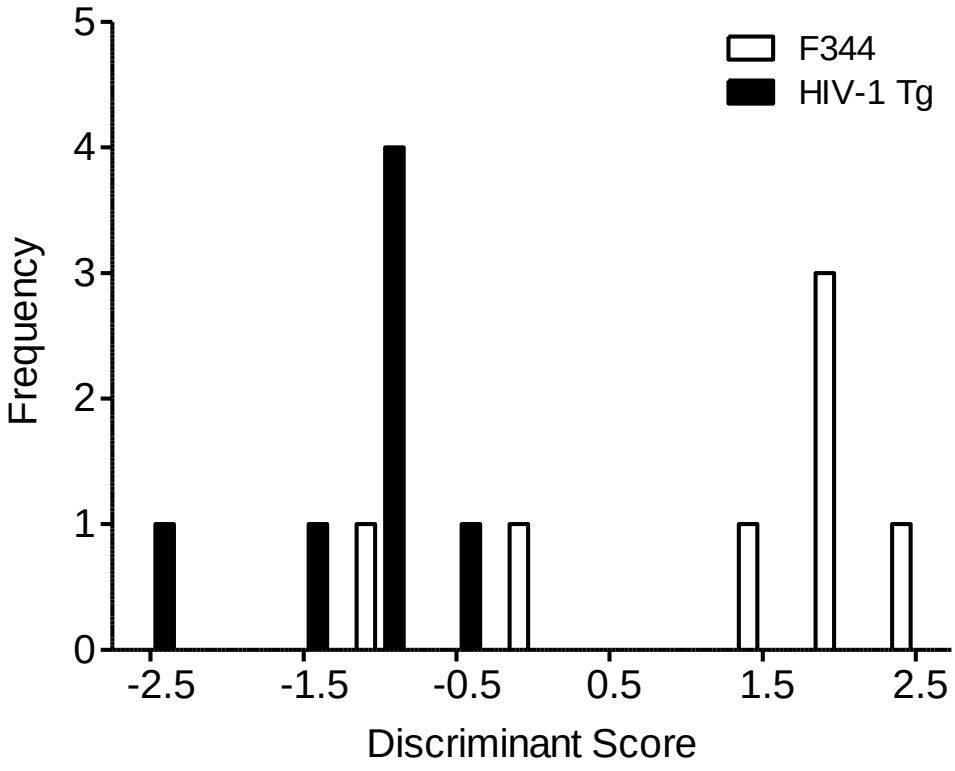

Figure 5.6 Discriminant function analysis. An exploratory discriminant function analysis was conducted to determine which neurochemical measures best differentiated group performance, and more critically, the extent to which the observed differences in measures correctly identified animals in regard to their group membership (HIV-1 Tg vs. F344 control). The predictor variables that were selected for the analysis were those neurochemical measures that were significantly predicted by behavioral measures in multiple regression analyses (PFC V_{max}, STR V_{max}, STR B_{max}, and DA turnover). Animal classification is illustrated as a function of the canonical variable representing the simplest linear function that best separated the HIV-1 Tg and control groups (canonical correlation of 0.82) and correctly classified the animals' group membership with 92.9% accuracy (100% of HIV-1 Tg animals, and 85.7% of controls; Wilks' $\lambda = 0.328$, $p \leq 0.05$).

Discussion

Psychostimulant abuse is highly comorbid with HIV-1 infection (Purohit et al., 2011; Ferris, Frederick-Duus, Fadel, Mactutus, & Booze, 2010)08), but it remains unclear how HIV-1 may impact the motivational processes which underlie drug abuse (Ferris et al, 2010). We found that the reinforcing efficacy of cocaine was diminished in the HIV-1 Tg rat. Specifically, although both HIV-1 Tg and control animals increased their cocaine intake over 14-days of PR responding, the HIV-1 Tg rats responded significantly less and maintained consistent low-level responding to cocaine (unit dose 1.0 mg/kg/infusion). There was no significant shift in EC50 for sucrose, although HIV-1 Tg rats responded significantly less for sucrose using both FR-1 and PR schedules of reinforcement. These findings suggest a profound effect on cocaine-maintained responding in the HIV-1 Tg rats, relative to sucrose responding. When animals were allowed a choice between cocaine and sucrose, animals initially preferred sucrose, but over time switched to a preference for cocaine. Thus, HIV-1 Tg rats exhibit disrupted choice behavior. DAT function was altered in the striatum of HIV-1 Tg rats; however prior cocaine self-administration produced a unique effect on dopamine homeostasis in the HIV-1 Tg rat, relative to the effects of either cocaine or HIV-1 alone. The alterations in reinforcing efficacy in the HIV-1 Tg rat resembles altered motivational states following HIV-1 infection (Paul et al., 2005a; Kamat et al., 2012; Castellon et al., 1998a). Our data suggest these alterations may reflect selective dysregulation within the dopaminergic circuitry, and represent a unique interaction of HIV-1 proteins with cocaine.

The dopamine transporter (DAT) may play a fundamental role in determining responding of HIV-1 Tg rats even in the absence of co-morbid drug exposure. There was a significant reduction in [^{3}H] WIN35,428 binding in the striatal synaptosomes derived from HIV-1 Tg animals when compared to F344 controls. This reduction in B_{max} HIV-1 Tg animals is consistent with our prior *in vitro* studies, which demonstrated that acute treatment with HIV-1 Tat protein reduced the membrane expression of the DAT (Midde et al., 2012), decreased [^{3}H]WIN 35,428 binding in rat striatal synaptosomes (Zhu et al., 2009) and decreased [^{3}H]WIN 35,428 binding in midbrain neurons *in vitro* (Aksenova et al., 2006). We found an increase in striatal Vmax for dopamine transport in the HIV-1 Tg rats. In sum, for the HIV-1 Tg rats, there was an increase in dopamine turnover in the striatum, reflecting that, although there were fewer DAT proteins on the membrane surface, these transporters were more efficient in dopamine clearance. This may represent a homeostatic process in which the HIV-1 Tg rat maintains consistent synaptic basal dopaminergic tone.

In contrast, dopamine homeostasis was differentially affected by cocaine self-administration in the HIV-1 Tg rats. In control animals, cocaine self-administration increased Vmax, which is an oft reported finding following repeated cocaine administration (Ramamoorthy et al., 2010; Oleson et al., 2009); however HIV-1 Tg animals which self-administered cocaine did not significantly increase Vmax, yet had similar [^{3}H]WIN35,428 binding as controls following cocaine. This resulted in decreased DA turnover in the striatum of the HIV-1 Tg rat following cocaine self-administration. We previously reported that direct infusion of the HIV-1 protein Tat into the NAc in animals with prior chronic cocaine experience produced a hyperdopaminergic basal tone

(Ferris et al., 2010) and altered behavior (Harrod et al., 2008). Specifically, the NAc of animals with prior cocaine experience had elevated basal DA levels when infused with Tat protein, but not following either cocaine or Tat alone; animals with Tat + cocaine treatments had approximately 400% greater extracellular DA levels (Ferris et al., 2010). Indeed, higher DA levels might be anticipated if the DAT function is compromised. Interestingly, acute treatment with Tat alone did not significantly alter DA levels, prior cocaine exposure was required. Collectively, these actions by HIV-1 following repeated cocaine administration may contribute to decreased DA turnover, subsequently increasing extracellular DA, but only with prior cocaine experience.

The HIV-1 Tg rat not only expresses Tat protein (Peng et al., 2010), but also gp120, Nef, and Rev viral proteins (without *gag-pol* genes, thereby rendering the rat non-infectious). HIV-1 protein expression in the HIV-1 Tg rat is under the control of the natural HIV-1 promoter, LTR, with protein expression in mononuclear phagocytes/astrocytes, but not in neurons (Royal, III et al., 2012) – a pattern similar to that observed in human HIV-1+ brains. Protein expression in the HIV-1 Tg rat is regulated by the viral LTR and uses rat cyclin-T as a cofactor to regulate Tat production (Reid et al., 2001). HIV-1 proviral DNA thereby produces viral proteins without active viremia. HIV-1 patients with latent HIV-1 infection (HIV-1+ without HIV-1 viral RNA or p24) may display mild to moderate cognitive impairment (Desplats et al., 2013). Tat protein is found in the CSF of aviremic patients (Johnson et al., 2013). Thus, without active infection, HIV-1 proviruses in the brain may produce viral proteins, and the production of these HIV-1 proteins is, for the most part, unaffected by antiretroviral drugs. Similar to Tat, gp120 protein has been found in HIV-1+ humans (Toneatto, Finco,

van der Putten, Abrignani, & Annunziata, 1999; Oh et al., 1992; Jones, Bell, & Nath, 2000) and gp120 exposure results in dopamine neuropathology *in vitro* (Hu, Sheng, Lokensgard, Peterson, & Rock, 2009; Wallace, Dodson, Nath, & Booze, 2006) and *in vivo* (Toggas et al., 1994). The HIV-1 Tg rat also produces gp120 (Peng et al., 2010). Thus, the HIV-1 Tg rat gives rise to a chronic exposure of the brain to HIV-1 viral proteins under the control of the natural promoter in disease appropriate cells. Thus, these proteins may be generated from proviral DNA without active infection; an aviremic state which may reflect protein expression in HIV-1 + patients on combined antiretroviral therapy (cART).

We found that sucrose had a lower reinforcing efficacy in the HIV-1 Tg rat. First, we used the sucrose preference task to assess changes in gustatory perception, and similar to a prior report (Peng et al., 2010), we found no difference in sucrose preference between HIV-1 Tg rats and controls. Second, HIV-1 Tg rats consistently responded for fewer sucrose reinforcers when compared to control rats, although there was no shift in the EC50 between groups. It is important to note that the animals were neither food nor water restricted during PR testing, so responses for sucrose were not affected by variations in satiety. Relative to mechanisms of cocaine reinforcement mechanisms, such as DAT function, alterations in sucrose reinforcement may reflect an impairment in either more widespread neural pathways (Berridge, Robinson, & Aldridge, 2009; Pennartz, Groenewegen, & Lopes da Silva, 1994) or dysfunction of a separate, discrete, neuronal system located within the NAc which is sensitive to sucrose and insensitive to cocaine (Carelli, 2002; Carelli & Wondolowski, 2006). As we found no shift in the EC50 for sucrose relative to controls, the neuronal systems underlying sucrose reinforcement

maybe less impaired in the HIV-1 Tg rats, relative to those for drug cocaine reinforcement.

It is interesting to compare the findings from the PR test with the choice task, as the two procedures may not assess the same motivational processes (Ahmed, Lenoir, & Guillem, 2013). All animals had extensive experience with both cocaine and sucrose reinforcement and were explicitly trained such that each lever had an equal likelihood of presenting either reinforcer (i.e., no bias). Importantly, cocaine preference was not attributed to a lack of interest in sucrose. Prior to the choice procedure, both cocaine and sucrose were consumed on several trials in which each reinforcer was presented alone. We found in the choice procedure, animals initially had a strong preference for choosing sucrose over cocaine. This is consistent with prior publications in which cocaine was found to have low value relative to natural rewards, such as sucrose, in rats (Cantin et al., 2010). Interestingly, over a period of days, most of the control animals shifted to a cocaine preference, suggesting an addictive process in that animals ultimately preferred to take cocaine at the expense of other available choices (i.e., sucrose); however, choice behavior was disrupted for the HIV-1 Tg rats. Additional research using choice procedures may determine if HIV-1+ drug abusers may be at particular risk for continued drug abuse or find psychostimulant drugs particularly rewarding.

The nucleus accumbens/ventral striatum has high levels of HIV-1 following infection (Wiley et al., 1998), decreases in volume following infection (Aylward et al., 1993; Paul et al., 2005b) and is a key area for the reinforcing actions of cocaine (Kalivas & Volkow, 2005). In particular, the medium spiny neurons (MSNs) of the NAc play a key role in many motivational (Enoksson, Bertran-Gonzalez, & Christie, 2012) and goal-

directed behaviors (Shen et al., 2009; Lesscher & Vanderschuren, 2012). We recently reported that the MSNs of the NAc are morphologically altered in HIV-1 Tg female rats (Roscoe, Jr., Mactutus, & Booze, 2014). MSNs in the NAc of HIV-1 Tg female rats had decreased dendritic branching and a population shift to shorter, less projected dendritic spines, relative to controls (Roscoe, Jr. et al., 2014) in response to chronic HIV-1 protein exposure. Chronic cocaine treatment is also known to significantly alter MSNs, primarily by increasing spine density and spine head diameter (Shen et al., 2009), as well as by bringing about increased synaptic strength (Robinson & Kolb, 1999; Shen, Moussawi, Zhou, Toda, & Kalivas, 2011). MSNs receive regulatory input from both the prefrontal cortex and the ventral tegmental area (Di Chiara & Imperato, 1988; Nestler, 2005); both of these afferents are modulated by cocaine. Given the apparent central role of MSNs in HIV-1 infection and cocaine abuse, it is possible that the MSNs represent structural loci (or part of key circuitry) for the interaction of HIV-1 proteins and cocaine on goal directed behaviors.

In sum, prior cocaine-exposure alters HIV-1 protein interactions with DA nerve terminals, producing unique motivational and neurochemical outcomes. We found, contingent upon prior cocaine self-administration, HIV-1 produces neurochemical alterations in the DAT with divergent outcomes relative to cocaine-free animals. The current study of synaptosomal DAT function/DA turnover in HIV-1 Tg animals which were self-administering cocaine, and our prior microdialysis study of HIV-1 Tat protein + cocaine (Ferris et al., 2010), suggests unique interactions between HIV-1 and repeated cocaine exposure. We reported that the HIV-1 Tat protein directly impairs DAT function (Zhu et al., 2009; Midde et al., 2013), produces oxidative damage in neurons (Aksenov et

al., 2006) and rat striatal tissue (Bansal et al., 2000; Aksenov et al., 2001). Similarly, the HIV-1 gp120 protein also impairs the function of the DAT in dopaminergic neurons (Hu et al., 2009) and produces oxidative damage (Agrawal et al., 2010; Yao, Allen, Zhu, Callen, & Buch, 2009; Wallace et al., 2006). The increased levels of synaptic dopamine, as a consequence of HIV-1 proteins and repeated cocaine co-exposure, may generate localized, low-level, oxidative stress, resulting in dysfunction of dopaminergic neurons following HIV-1 infection. Over a prolonged period of exposure to HIV-1 proteins and cocaine, loss of dopaminergic nerve terminals, and ultimately, dopaminergic cell loss may occur, as has been reported in HIV-1+ human drug abusers (Kumar et al., 2011). Our results suggest that DAT functions in the face of HIV-1+cocaine challenge may be fundamental in determining the expression of goal-directed behaviors for cocaine in the HIV-1 Tg rat. The goal of maintaining DAT functionality in the presence of cocaine and HIV-1 proteins may lead to effective and uniquely targeted therapeutics for the HIV-1+ cocaine abuser.

CHAPTER 6

A POTENTIAL GUT MICROBIOME THERAPEUTIC APPROACH FOR HIV-1+ DRUG ABUSERS[5]

[5] SJ Bertrand, CF Mactutus, SB Harrod, RF Roscoe Jr., RM Booze. To be submitted to Journal of NeuroImmune Pharmacology.

Introduction

The use of illicit drugs, like cocaine, compound peripheral and neurological complications of HIV-1 (Baum et al., 2009; Chang et al., 2008; Nath et al., 2001; Nath, 2010). HIV-1+ cocaine users progress from HIV-1 to AIDS more quickly (Fiala et al., 1998; Baum et al., 2009), and are more likely to display neurocognitive disorders than their non-abusing cohorts (Devlin et al., 2012; Weber et al., 2013; Levine et al., 2006; Gongvatana et al., 2014; Nath et al., 2001). Cocaine use in conjunction with HIV-1 is also associated with decreased medication adherence (Panos et al., 2014), increased viral load both as a result of poor medication adherence (Qian et al., 2014) and independent of antiretroviral therapy (ART) compliance (Rasbach et al., 2013). Presently there are no therapeutics for HIV+ drug users, or HIV-1 associated neurocognitive disorders (HAND) in general. Addressing HIV-1+ drug use may result in a reduction in disease transmission, an increase in medication adherence and decreased viral load (Roux et al., 2008), resulting in a higher quality of life and decreased mortality.

HIV-1+ individuals have an increased incidence of apathy, or a lack of motivation (Kamat et al., 2012; Rabkin et al., 2000; Tate et al., 2003). Clinically apathetic HIV-1+ individuals are more likely to exhibit neurocognitive deficits (Paul et al., 2005a; Rabkin et al., 2000; Shapiro et al., 2013), decreased NAc volume (Paul et al., 2005b), and a poorquality of life (Tate et al., 2003). HIV-1 targets the dopamine system (Agrawal et al., 2010; Gelman et al., 2006; Kumar et al., 2009a), which plays a major role in maintaining and initiating motivated behavior (Baik, 2013); and the dopaminergic system is believed

to be ‘hijacked’ by drugs of abuse through long term morphological changes (Shen et al., 2009; Toda, Shen, Peters, Cagle, & Kalivas, 2006; Nestler, 2005). Individuals with HAND who use cocaine have decreased dopamine transporter (DAT) levels in the striatum (Chang et al., 2008), decreased TH staining at autopsy (Silvers et al., 2006) and increased neuronal damage (Gill & Kolson, 2014; Martin-Thormeyer & Paul, 2009). HIV-1 and stimulant drugs act in concert to produce a greater risk for neurocognitive disorders and apathy, indicating a need for therapeutic intervention.

The HIV-1 transgenic (HIV-1 Tg) rat has been used to study the neurological effects of HIV-1 proteins without active viral replication (Moran, Booze, Webb, & Mactutus, 2013; Moran et al., 2014; Moran et al., 2013), similar to an individual on antiretroviral therapy (ART) (Peng et al., 2010). The HIV-1 Tg rat expresses 7 of the 9 HIV-1 genes. The virus lacks the genes *gag* and *pol,* and thus is not replicative (Reid et al., 2001). Neurotoxic proteins gp120 and Tat proteins, which both interact with stimulant drugs to produce enhanced neurological insult (Bansal et al., 2000; Aksenov et al., 2006), are present in the brain of the HIV-1 Tg rat (Rao et al., 2011). There is significant dopaminergic dysfunction (Moran et al., 2013; Moran et al., 2012; Webb, Aksenov, Mactutus, & Booze, 2010), and altered response vigor for sucrose and cocaine in the HIV-1 Tg rat (See chapter 5). Although the HIV-1 Tg rat does not fully mimic the pathological hallmarks of HIV-1, it does provide a solid model of neurological complications that occur due to chronic exposure to HIV-1 proteins and provides the opportunity to test potential therapeutics for neurocognitive dysfunction.

The soy derived phytoestrogen daidzein prevents synaptodendritic damage and restores the F-actin rich network after exposure to HIV-1 Tat (Bertrand et al., 2014).

During digestion, daidzein is metabolized by the gut bacteria to produce S-equol (Setchell et al., 2005). Equol is 50-70 fold more potent than its parent compound (Jiang et al., 2013b), and S-equol has a 24-fold binding specificity for estrogen receptor subtype beta (ERβ) over estrogen receptor subtype alpha (ERα) (Jiang et al., 2013b). ERβ activation has been shown to attenuate HIV-1 Tat induced cell death (Adams et al., 2010) and synaptodendritic injury (See chapter 4). Importantly, both enantiomers of equol successfully prevent interactive damage produced by HIV-1 Tat and cocaine through an ERβ dependent mechanism (see chapter 4). However the effects of equol on motivation and/or drug abuse or HIV-1 have not been studied.

HIV-1 is associated with dendritic alterations *in vivo* (Masliah et al., 1997; Moore et al., 2006; Sa et al., 2004), and these alterations are predictive of cognitive performance (Moore et al., 2006; Adle-Biassette et al., 1999). The HIV-1 Tg rat has alterations in temporal processing and deficits in executive function tasks (Moran et al., 2014; Moran et al., 2013), in addition to altered dendritic spine morphology (Roscoe, Jr. et al., 2014), suggesting that altered neuronal structure may play a role in behavioral changes of the HIV-1 Tg rat. Exposure to cocaine and other stimulant drugs often results in an increase in spine density of medium spiny neurons (MSNs) in the nucleus accumbens (NAc) (Robinson & Kolb, 2004; Grueter, Robison, Neve, Nestler, & Malenka, 2013; Russo et al., 2010). On the other hand, HIV-1 proteins produce a decrease in overall spine density *in vitro* (Atluri, Kanthikeel, Reddy, Yndart, & Nair, 2013; Atluri et al., 2014; Tovar et al., 2013; Kim et al., 2008a) and *in vivo* (Fitting et al., 2010b; Fitting et al., 2013). The effects of HIV-1 and cocaine in combination on dendritic spine parameters are difficult to predict. Since dendritic injury occurs prior to cell death in HIV-1 and is potentially

reversible, it is an important marker for therapeutic potential of compounds in HIV-1 associated neurocognitive disorders and stimulant abuse.

Estrogen is a well-known proliferator of dendritic spine density (Phan et al., 2011; Gould et al., 1990; Woolley & McEwen, 1992), and a potent neuroprotective agent (Simpkins et al., 2012; Simpkins & Singh, 2008). Although both traditional ER subtypes (α,β) participate in the modulatory actions of estrogen, selective activation of ERβ increases spine density and PSD95 accumulation (Srivastava et al., 2010). ERβ activation prevents HIV-1 Tat induced neuronal apoptosis (Adams et al., 2010), and synaptodendritic damage produced by HIV-1 Tat and cocaine *in vitro* (See chapter 4). Together, these results suggest that ERβ activation, through the use of a subtype selective compound (e.g., equol), would be a beneficial therapeutic for HIV-1+ drug users.

We used the HIV-1 Tg rat to evaluate the therapeutic potential of oral administration of equol in ameliorating the alterations in response vigor displayed by the HIV-1 Tg rat. Sucrose and IV cocaine self-administration paradigms were used to evaluate the effectiveness of equol treatment in modifying motivated behavior. Following behavioral testing, the impact of equol treatment and HIV-1 on medium spiny neurons (MSNs) in the NAc was evaluated using DiOlistic labeling and confocal microscopy. The results suggest that equol treatment results in long-term behavioral and morphological changes, particularly in control F344 rats.

Methods

Animals

A total of 42 adult female ovariectomized (OVX) (21 HIV-1 Transgenic and 21 F344 control) rats were purchased from Harlan Laboratories, Inc. (Indianapolis, IN,

USA). All animals were ovariectomized due to the potentially confounding effects of estradiol. Estradiol potentiates the reinforcing efficacy of cocaine (Hu et al., 2004) and prevents HIV-1 mediated neuronal damage (Bertrand et al., 2014; Adams et al., 2010; Heron et al., 2009), and suppresses HIV-1 transcription (Cabrera-Munoz et al., 2012). Additionally, estradiol effects may have confounded equol treatment effects. Ovariectomies were performed at Harlan Laboratories prior to arrival at the University of South Carolina. Rats were fed a minimal phytoestrogen diet (≤20 ppm of phytoestrogen; Teklad 2020X Global Rodent Diet; Harlan Laboratories, Inc., Indianapolis, IN). Standard rodent chow contains 200-500ppm soy and alfalfa (Harlan Laboratories, Inc., USA). Soy and alfalfa contain phytoestrogens, compounds which have estrogenic effects due to their natural structural similarity to estrogen (Lephart et al., 2005; Setchell & Cassidy, 1999). Additionally, soy contains the compound daidzein which is converted into s-equol, the experimental compound, by the gut microbiota (Setchell & Cassidy, 1999; Setchell et al., 2005). Throughout the experiment animals had *ad libitum* access to food and water, unless otherwise specified. The animal colony was maintained at 21±2˚C, 50±10% relative humidity and a 12L:12D cycle with lights on at 0700h. The Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina (animal assurance number: A3049-01) approved all experimental procedures involving animals.

Apparatus

Sound-attenuating enclosures housed operant chambers (ENV-008; Med-Associates, St. Albans, VT, USA) which were controlled by Med-PC computer interface software. Front and back panels were composed of stainless steel, the sides and top of the chamber consisted of polycarbonate. The front stainless steel panel contained a magazine that allowed a recessed 0.01cc dipper cup (ENV-202C) to deliver a solution through a

5cm x 5cm opening following completion of a response requirement (ENV 202M-S). Two retractable active (i.e., response resulted in reinforcement) metal levers (ENV-112BM) on either side of the receptacle were located 7.3 cm above a metal grid floor. A 28-V white cue light, 3 cm in diameter, was located above each active response lever but never illuminated. Head entries into the magazine were detected using an infrared sensor (ENV 254-CB). There was a non-retractable lever ("inactive"; responses made on this lever were recorded but not reinforced) located on the center back panel and a 28V house light was located above the lever. A syringe pump (PHM-100) was used to deliver intravenous cocaine infusions through a water-tight swivel (Instech 375/22ps 22GA; Instech Laboratories, Inc., Plymouth Meeting, PA), which was connected to the backmount of the animal using Tygon tubing (ID, 0.020 IN; OD, 0.060 IN) enclosed by a stainless steel tether (Camcaths, Cambridgeshire, Great Britain). The pump infusion times were calculated by a Med-PC computer program according to the animal's bodyweight, which was updated daily.

Drugs

In order to prevent significant hydrolysis, cocaine solutions were prepared prior to the start of each operant test. Cocaine hydrochloride (Sigma-Aldrich Pharmaceuticals, St. Louis, MO) was weighed as a salt and was dissolved in physiological saline (0.9%; made fresh daily). Sucrose solutions were prepared fresh at the beginning of each testing day. Heparin was purchased from APP Pharmaceuticals (Schamburg, IL), butorphenol (Dolorex) from Merck Animal Health (Millsboro, DE), Sevofluorane, USP from Baxter (Deerfield, IL), and Gentamicin sulfate from VEDCO (Saint Joseph, MO). Equol and sucrose pellets (100 mg; banana flavor) were purchased from Bio-Serv (Flemington, NJ, USA). Equol was purchased from Cayman Chemical (Ann Arbor, MI, USA) and

formulated into 100 mg sucrose pellets (0.05 mg equol/pellet) by Bio-Serv prior to arrival at the University of South Carolina. Pellets were color coded (equol: orange; sucrose: yellow) to avoid experimenter confusion.

Treatment

Each genotype was randomly assigned to one of two treatment groups: Sucrose (*n*=10 F344-S and *n*=10 HIV-1-S) and equol (*n*=11 F344-E and *n*=11 HIV-1-E). Treatment consisted of 0.2 mg equol (4 pellets) or 4 sucrose control pellets per animal. Oral dosing of equol (0.2mg) used is within the dose range used in human studies (~10-30mg for ~60kg human) (Jackson et al., 2011; Setchell, Zhao, Shoaf, & Ragland, 2009). Animals were treated once daily for one week prior to the start of testing, and every day thereafter until catheterization. Animals did not receive treatment for one week following surgery. When treatment resumed, it was given every other day until the end of the 14-day cocaine self-administration progressive ratio task. No treatment occurred during the cocaine dose-response or choice tasks. During sucrose tasks animals were pair-housed in their home cages. To ensure individual treatment consumption, animals were placed into separate, empty cages until designated pellets were consumed. Following catheterization, animals were single housed, and thus were given treatment in their home cage.

Experiment 1: Sucrose-Maintained Responding

Previously established research protocols were used to determine dipper training and auto-shaping (Harrod, Lacy, & Morgan, 2012b; Lacy, Hord, Morgan, & Harrod, 2012b; Reichel, Linkugel, & Bevins, 2008). Briefly, during dipper training all animals learned to approach the magazine and drink from the dipper. Next, animals learned to respond for 5% sucrose (w/v) during autoshaping. Animals were water restricted for 12-15 hours prior to dipper training, and auto-shaping. During the auto-shaping task two

retractable active levers (responding was recorded and reinforced) were present at the front of the chamber. During all phases of the experiment active levers were programed to retract if there was a difference of 5 or more responses on either lever in order to control for side bias. In the rear center panel of the chamber there was an inactive lever on which responses were recorded but not reinforced. After completion of the daily operant task, animals were given free access to water in their home cage for 9-12 hours. Once animals met the criterion of 60 or more reinforcers for 3 consecutive days, animals had *ad libitum* access to water and continued onto experiment 1.1.

Experiment 1.1

Due to auto-shaping, animals were experienced with the fixed ratio (FR) schedule of reinforcement and did not require new training in order to participate in the progressive ratio (PR) or FR phases of the experiment. After 1 maintenance day (5%; FR1) the schedule of reinforcement was changed to PR (max. 120 minute sessions). During PR tests responding on either active lever contributed to meeting the ratio requirement; once the ratio requirement was met, animals had 4s access to sucrose reinforcement. Ratios progressively increased, according to the following function: $[5e^{(\text{reinforcer number X } 0.2)}]$-5 (rounded to the nearest integer) (Richardson & Roberts, 1996). One of five sucrose concentrations (1%,3%,5%,10%, and 30% w/v; Latin square procedure) was presented as the reinforcer on the test days, which occurred every other day. Maintenance trials occurred on non-test days, which made 5% sucrose available on an FR1 schedule of reinforcement. Water was presented as the sixth and last reinforcer for all rats in order to prevent potential extinction learning if water was not reinforcing to the animals.

Experiment 1.2

Following completion of the PR sucrose paradigm, animals were given the opportunity to respond for identical sucrose concentrations (1-30%) on an FR1 schedule of reinforcement. Testing days were every other day, with a 5% maintenance day on the intervening days. Water was presented as the sixth and last reinforcer for all rats.

Experiment 2: Cocaine maintained responding

Surgery

Following the completion of experiment 1, IV catheters were implanted into all animals. Catheterization was performed according to Smith et al, (2014). Anesthesia was induced using 5% inhalant sevofluorane and animals were maintained at 3.5-4% sevofluorane throughout the surgical procedure. After anesthesia induction, a sterile IV catheter was implanted into the right jugular vein and secured with 4-0 Perma-Hand silk sterile sutures (EthiconEnd-Surgery, Inc.). The catheter extended dorsally, where it was connected to an acrylic pedestal embedded with mesh. The catheter pedestal was implanted subcutaneously above the right and left scapulae. The back-moiunt was stitched into place using Sterile 4-0 Monoweb sutures. In order to provide post-operative analgesia and prevent infection, all animals were administered butorphenol (0.8 mg/kg, s.c.) and gentamicin (0.2 ml 1%, I.V.) immediately following surgery. All rats were monitored in a heat-regulated warm chamber following surgery and returned to the colony room after recovery from anesthesia. For one week following surgery catheters were flushed daily with a solution containing the anti-coagulant heparin (2.5%) and the antibiotic gentamicin (1%) to prevent clotting and infections. Catheters were flushed with 0.9% saline solution prior to testing each day and flushed again with post-flush solution

after operant testing. Cocaine testing began and equol treatment resumed 7 days post-surgery. Two HIV-1 Tg animals died immediately following surgery; one of unknown causes and one suffered from a seizure after returning to the home cage but prior to returning to the colony room.

Cocaine-maintained responding

There were three phases to this experiment. The first two phases (experiments 2.1 and 2.2) of this experiment utilized a modified version of the Morgan et al., (2006) cocaine self-administration procedure that was employed previously (see chapter 5). During experiment 2.1, rats responded for cocaine according to a daily FR1 schedule of reinforcement (0.2 mg/kg/inj) for 5 consecutive days (1 hour sessions); once the response requirement was complete a 20s time-out occurred, i.e. active levers retracted and the house light was extinguished. Next, during experiment 2.2 they were switched a higher concentration (0.75 mg/kg/inj) of IV cocaine using a PR schedule of reinforcement ($[5e^{(\text{reinforcer number X } 0.2)}]$-5; rounded to the nearest integer) (Richardson & Roberts, 1996) for a maximum of 120 minutes for 14 consecutive days. Upon ratio completion there was a 20s time-out. Finally, experiment 2.3 consisted of a PR paradigm with ascending doses of cocaine (0.01, 0.05, 0.1, 0.2, 0.75, and 1.0 mg/kg/inj) across sessions to evaluate potential changes in sensitivity and reinforcing efficacy of cocaine induced by genotype and/or equol treatment. Following completion of the ratio requirement a 20s time-out occurred. Maintenance trials using the training dose (0.2 mg/kg/inj) on a FR1 schedule occurred every other day during the dose-response paradigm.

Experiment 3: Choice Behavior

All four groups of animals were subjected to a choice self-administration protocol following completion of the cocaine experiments. The training dose for sucrose (5%) and

cocaine (0.2mg/kg/inj) were used as the concentrations for choice behavior. The training doses were used due to a lack of clear EC50 in the cocaine or sucrose data for any group. Four forced choice trials, with only one lever available during these trials, began each session (2 nondrug and 2 drug reinforcers) followed by the choice component. Sucrose paired lever presentation (right or left) was balanced between groups. Following the forced choice trials, both levers were concurrently available to allow the animals to freely choose between sucrose and cocaine. Once a response was made, a 20s time out occurred between responses. During all studies there was an inactive back lever located in the center back panel of the chamber. Responses on the back lever were recorded but not reinforced.

Experiment 4: Dendritic Spine Analysis

Preparation of Tissue

Animals were sacrificed within 24 hours of their last self-administration session. First, animals were deeply anesthetized using sevofluorane (Abbot Laboratories, Chicago IL, USA) and then transcardially perfused with 100 ml of 100 mM PBS wash followed by 100-150 ml 4% paraformaldehyde buffered in PBS (Sigma-Aldrich, St. Louis, MO, USA). After dissection, brains were post-fixed in 4% paraformaldehyde for 10 minutes and then cut into 200 µm thick coronal slices using a rat brain matrix (ASI Instruments, Warren, MI, USA). Serial slices were then washed 3x in PBS, notched for orientation, and placed into tissue culture plates (24 well plate; Corning, Tewksbury MA) until further processing.

Preparation of DiOlistic Cartridges

DiOlistic labeling was performed according to published techniques (Roscoe, Jr. et al., 2014; Seabold, Daunais, Rau, Grant, & Alvarez, 2010). Tungsten beads (~300mg; Bio-Rad, Hercules, CA, USA) were dissolved in 99.5% pure methylene chloride (Sigma-Aldrich, St. Louis, MO, USA) and sonicated in a water bath for 30 minutes (37°C). Methylene chloride was also used to dissolve crystalized DiI (14.5 mg; Invitrogen Life Sciences, Carlsbad, CA, USA). After sonication, the bead solution (100 µl) was placed on a glass slide and the DiI solution (150 µl) was titrated on top and mixed using a pipette tip. The mixture was then allowed to air dry. A razor blade was used to collect and transfer the dried dye/bead mixture onto wax-coated weigh paper. Finally, the dye/bead mixture was transferred to a 15 ml conical tube (BD Falcon, San Jose, CA, USA) with 3 ml ddH_2O and was then sonicated for 45-60 minutes.

Preparation of Tefzel Tubing

Polyvinylpyrrolidone (PVP, 100mg; Sigma-Aldrich, St. Louis, MO) was dissolved in 10 ml ddH_2O, vortexed, and then passed through three 1.7 m lengths of Tefzel tubing (IDEX Health Sciences, Oak Harbor, WA, USA). Subsequently, 3 ml bead/dye solution was slowly drawn into the tubing and placed into the tubing prep station (Bio-Rad, Hercules, CA) for 5 min. Water was drained from the tube and the dry tubing was spun for approximately 10 min with a nitrogen gas flow of 1.0LPM in the tubing prep station. Then the nitrogen gas flow through the tubing was adjusted to 0.4-0.5 LPM and the tubing was spun for an additional 50-60 min. Finally, dry tubing was cut into 13 mm segments and stored under anhydrous conditions until use.

DiOlistic Labeling using the Helios Gene Gun and Immunohistochemistry

The Helios gene gun (Helium gas 80 PSI; Bio-Rad, Hercules, CA, USA) was loaded with previously prepared cartridges, and particles were delivered through 3 µm pore filter paper directly onto the slice with the barrel placed approximately 2.5 cm away from the sample. Sections were washed 3x in PBS and stored overnight at 4°C to allow dye diffusion. Tissue sections were mounted using Pro-Long Gold Antifade (Invitrogen Life Sciences, Carlsbad, CA, USA), coverslipped (#1 cover slip; Thermo Fisher Scientific, Waltham MA, USA) and stored in the dark at 4°C.

Medium Spiny Neuron Dendritic Analysis and Spine Quantification

MSNs were analyzed from the NAc, located approximately 2.28 mm to 0.60 mm anterior to Bregma (Paxinos & Watson, 2007). For dendritic branch order analysis 1-4 MSNs per animal were randomly selected. Branch order was only determined for animals with clear dendritic arbor as assessed by the maximum intensity projection image (F344-S: n=14; F344-E: n=15; HIV-1-S: n=11; HIV-1-E: n=15). MSNs in the NAc were used for spine analysis provided the following criteria were satisfied: 1. Continuous staining beginning in the cell body extending throughout the dendrite, 2. Minimal DiI diffusion, and 3. Low background fluorescence. Each animal had 3-8 MSNs that were randomly selected for spine analysis. Numbers of individual neurons per group were F344-S: 36, F344-E: 39, HIV-1-S: 30, and HIV-1-E: 44.

Z-stack images were obtained using a Nikon-TE 2300T confocal microscope with Nikon's EZ-C1 software (version 3.81b). Dendritic spine analysis and branch order analysis was performed at 60x (n.a.=1.4) with Z-plane intervals between 0.15-0.25 µm (pinhole size 30 µm; back-projected pinhole radius 167 nm). For DiI fluorophore excitation, a green helium-neon (HeNe) laser with an emission of 533 nm was used.

Neurolucida version 10.52, equipped with AutoNeuron and AutoSpine extension modules (MicroBrightField, Williston, VT, USA) was used to analyze dendritic spine parameters.

Dendritic Spine Parameters

Dendritic spine parameters of length, volume, and head diameter were analyzed. Spine length was defined to be between 0.01 to 4 µm; lengths greater than 4 µm were considered to be filopodia and excluded from the study (Blanpied & Ehlers, 2004; Ruszczycki et al., 2012). Spine volume parameters were defined as those measures between 0.02 and 0.2 μm^3 (Merino-Serrais et al., 2013). Spine head diameters were defined as those measures between 0.3 and 1.2 µm (Bae, Sung, Cho, Kim, & Song, 2012).

Statistical Analysis

Data analyses were performed using SPSS version 22 (IBM Corporation, Armonk NY) and Graphpad version 5.02 (Graphpad Software, Inc., La Jolla, CA). For experiment 1, a mixed-design analysis of variance (ANOVA) was used to analyze to performance on an FR and PR schedule of reinforcement with genotype (HIV-1 Tg vs. F344 control) and treatment (Equol vs. Sucrose) as the between-subjects factors and sucrose concentration (1-30%, w/v) as the within-subjects factors and obtained sucrose reinforcers as the dependent variable. Experiment 2 was also analyzed using a mixed-design ANOVA, with genotype and treatment as the between-subjects factors and day or concentration as the within-subjects factors and obtained reinforcers as the dependent variable. For analysis of the choice paradigm, a mixed-design ANOVA was used to analyze choice results with genotype, treatment, reinforcer type (Sucrose vs. Cocaine) as the between-subjects

factors and day as the within-subjects factor with active presses as the dependent variable. Curve fit and regression analyses were used to detect individual group differences revealed by the overall ANOVA. For analysis of dendritic spine parameters, a two-way ANOVA was utilized with each spine parameter (length, diameter, volume, or density) as the dependent variable, and treatment and genotype as the between-subjects variables. Statistical comparisons were made using ANOVA techniques with a priori contrasts to determine specific treatment effects. For analysis of cocaine data, animals with obvious patency issues (e.g. leak at base of back mount or inability to flush) were excluded from the analysis. Significant differences were set at $p \leq 0.05$.

Stepwise regression was used to detect any significant behavioral predictors of morphological changes in the NAc. Models were considered significant if $p \leq 0.05$.

Results

Experiment 1.1: Sucrose PR responding

Animals were treated with 0.2 mg equol formulated into sucrose pellets or identical sucrose pellets without equol daily for one week prior to the start of the experiment, and daily for the duration of experiment 1. Following successful completion of an auto-shaping task (60+ reinforcers/3 consecutive days), HIV-1 Tg and F344 rats were given the opportunity to respond on a progressive ratio schedule of reinforcement for 1, 3, 5, 10, and 30% (w/v) sucrose solutions. A 2 x 2 x 5 mixed ANOVA using number of active lever presses as the dependent variable was used to examine these data. Planned contrasts revealed significant linear effect of concentration $F(1,34)=8.3$, $p \leq 0.01$, with all animals having an increased response for higher concentrations of sucrose (figure 6.1A). There was a significant main effect of genotype $F(1,34)=4.0$, $p \leq 0.05$, with HIV-1

Tg rats responding less overall compared to F344 control rats across all concentrations, regardless of treatment (figure 6.1A).

Experiment 1.2: Sucrose FR responding

During the second phase of experiment 1, animals were given the opportunity to respond on a FR1 schedule of reinforcement for the same sucrose concentrations used in phase 1. A 2 x 2 x 5 mixed ANOVA with reinforcers received as the dependent variable was used to examine these data. Similar to experiment 1.1, there was a prominent linear effect of concentration $F(1,24)=103.2$, $p\leq0.001$, with animals across genotypes responding with increased vigor as concentration increased. There was a significant main effect of genotype $F(1,24)=9.0$, $p\leq0.02$, with HIV-1 Tg rats receiving fewer sucrose reinforcers than F344 rats (figure 6.1B). There was a significant main effect of treatment $F(1,25)=7.9$, $p\leq0.01$, with equol treated animals responding significantly more than sucrose treated animals on concentrations 1-10%. There were significant concentration x genotype $F(4,96)=4.2$, $p\leq0.02$ and concentration x treatment $F(4,96)=3.4$, $p\leq0.05$ interactions. Planned contrasts revealed a significant quadratic interaction between concentration and treatment $F(1,24)=6.3$, $p\leq0.05$, and a significant cubic effect of concentration by genotype $F(1,24)=9.4$ $p\leq0.01$.

A curve fit analysis was used further explore the significant concentration x treatment interaction. A one phase association curve was a significant fit for equol treated animals ($r^2=0.98$), while sucrose treated animals had a significantly linear fit ($r^2=0.99$) (figure 6.1C). These fits were significantly different from one another $F(1,90)=6.7$, $p\leq0.01$, and indicate that equol treatment results in a response plateau between the highest sucrose concentrations (10% and 30%).

A curve fit analysis was also used to evaluate the genotype x concentration interaction. F344-E animals had a significantly different fit than F344-S animals in experiment 1.2, $F(1,37)=7.8$, $p\leq0.01$. A one phase association curve was found to significantly fit F344-E ($r^2=0.99$), alternatively, there was a significant linear fit to the F344-S animals ($r^2=0.95$) (Figure 6.1E). HIV-1-S ($r^2=0.98$) and HIV-1-E ($r^2=0.89$) followed a significantly linear pattern, however HIV-1-E rats received significantly more sucrose reinforcers than HIV-1-S rats, as demonstrated by a significantly higher intercept $F(1,92)=3.8$, $p\leq0.05$ (figure 6.1D).

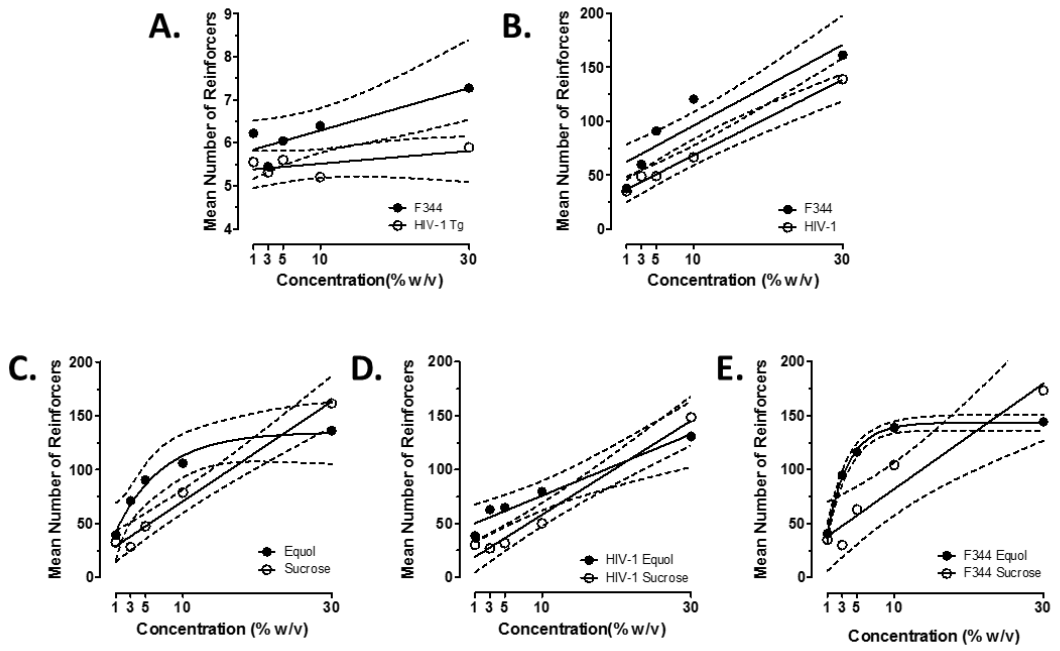

Figure 6.1 The HIV-1 Tg rat demonstrates diminished response vigor for sucrose and S-equol functionally alters sucrose response. **A.** F344 and HIV-1 animals had a significantly linear response, $p\leq0.01$, to increasing concentrations of sucrose (1-30%; w/v) using a progressive ratio schedule of reinforcement. There was a main effect of genotype, with HIV-1 Tg rats responding less overall ($p\leq0.05$) regardless of concentration. **B.** F344 animals respond more overall for sucrose on an FR1 schedule of reinforcement $p\leq0.02$, however both groups display a linear response to increasing concentrations of sucrose (1-30%; w/v), $p\leq0.001$. **C.** Equol treatment results in a functionally different response to increasing concentrations of sucrose. Equol treatment resulted in a quadratic response to sucrose; there was a significant plateau of responding

between 10% and 30% (w/v) sucrose (one phase association curve; r^2=0.98). Meanwhile sucrose treatment resulted in a functionally linear response for increasing concentrations of sucrose (linear fit; r^2=0.99). **D.** Equol treatment did not significantly alter the linear response for sucrose in the HIV-1 Tg rat; although it did result in a significantly higher intercept, $p \leq 0.05$, suggesting HIV-1-E animals respond more for sucrose than HIV-1-S animals. **E.** F344-E animals have a significant fit of a one phase association curve (r^2=0.99) while F344-S rats have a significant linear fit (r^2=0.95). Data represents mean values ± 95% CI.

Experiment 2.1: Cocaine acquisition

Animals were catheterized and allowed to heal for one week prior to testing. A mixed 2 x 2 x 5 ANOVA was used to analyze the data obtained during the 5 day acquisition period. There was a significant linear effect of day $F(1,36)=9.0$, $p \leq 0.01$, and a significant day x treatment interaction $F(4,144)=3.1$, $p \leq 0.05$. Regression analyses confirmed that equol treated animals, regardless of genotype, had a negative slope significantly different from zero $F(1,196)=4.1$, $p \leq 0.05$ across the 5 days of acquisition when compared to sucrose treated animals; on the other hand, sucrose treated animals had a slope that was not significantly different from zero (figure 6.2A).

Experiment 2.2: 14 Day Cocaine PR

Following experiment 2.1, a 14 day progressive ratio task was used to evaluate the ability of the animals to escalate their cocaine intake. A 2 x 2 x 14 mixed ANOVA was initially used to evaluate these results. There was a significant main effect of day $F(13,364)=2.0$, $p \leq 0.02$, suggesting that all animals increased their drug intake over the 14 day testing period. Linear regression analyses confirmed that the slope of the lines for both HIV-1 Tg and F344 animals were positive and significantly different than zero $F(1,12)=28.8$, $p \leq 0.001$ and $F(1,12)=35.3$, $p \leq 0.001$, respectively.

Linear regression analyses also revealed that there was not a significant genotype difference between slopes. However HIV-1 Tg rats (r^2=0.70) had a significantly different

intercept than F344 (r^2=0.75) rats $F(1,557)=5.2$, $p\leq0.02$, indicating that HIV-1 Tg rats received more infusions overall but escalated at the same rate as F344 rats (figure 6.2B). Furthermore, F344-E treated rats (r^2=0.68) had a significantly different intercept compared to F344-S (r^2=0.50) rats $F(1,291)=16.6$, $p\leq0.001$ but not a significantly different slope suggesting that F344-E rats responded more than F344-S treated rats but both groups escalated at the same rate (figure 6.2C). Conversely, HIV-1-E rats (r^2=0.50) had a significantly different intercept than HIV-1-S (r^2=0.69) rats $F(1,263)=7.1$, $p\leq0.01$, but a similar slope, indicating HIV-1-S and HIV-1-E rats increased intake at the same rate but HIV-1-E rats received fewer cocaine reinforcers than HIV-1-S rats (figure 6.2D).

Experiment 2.3: Cocaine Dose-Response

Following experiment 2.2, animals were tested on 6 doses of cocaine (0.01-1mg/kg/infusion) on a PR schedule in an ascending manner. Testing occurred every other day, with a maintenance day (FR) on the intervening days. Animals were no longer given equol or sucrose pellets. A 2 x 2 x 6 ANOVA with number of earned infusions as the dependent variable was employed initially to examine these data. There was a significant main effect of dose $F(5,95)=6.4$, $p\leq0.003$, which was found to be a linear effect using planned contrasts $F(1,19)=9.4$, $p\leq0.006$. There was a significant main effect of genotype $F(1,19)=5.2$, $p\leq0.05$, which HIV-1 Tg animals receiving fewer cocaine infusions overall. There was also a significant dose x genotype interaction $F(5,95)=2.9$, $p\leq0.05$.

A linear regression was used to examine the effects of genotype on the slope of the line, F344 animals had a robust linear fit (r^2=0.96) while HIV-1 Tg animals only had a moderate fit (r^2=0.44) and the slopes were significantly different from one another $F(1,134)=4.6$, $p\leq0.03$. Only F344 animals had a slope significantly different from zero $F(1,58)=11.7$, $p\leq0.001$, indicating that HIV-1 Tg animals did not vary in responding to increasing doses of cocaine (figure 6.2E). Prior experience with equol did not produce a significant effect in either HIV-1 Tg or F344 animals.

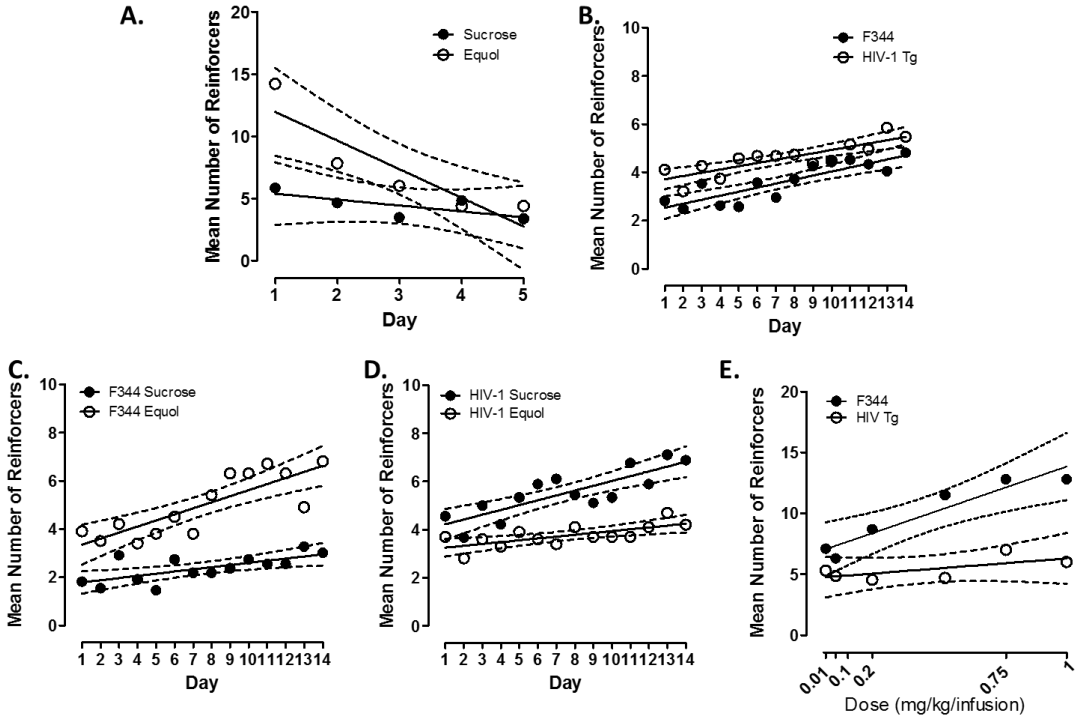

Figure 6.2 Equol treatment differentially modulates response for cocaine according to genotype. **A.** During the acquisition phase, equol treated animals, regardless of genotype, have a significantly negative slope $p\leq0.05$, while sucrose treated animals have a slope that is not significantly different from zero. Equol treated animals initially took more cocaine than sucrose treated animals, but eventually fell to similar levels of responding. **B**. During the 14 day PR task, HIV-1 Tg animals and F344 animals both have non-zero, but similar, slopes ($p\leq0.001$), indicating that both groups of animals escalated cocaine intake at the same rate. However, HIV-1 Tg animals have a significantly higher intercept $p\leq 0.02$ demonstrating that HIV-1 Tg animals took more cocaine overall. **C.** F344-E and

F344-S animals had similar slopes, but different intercepts ($p \leq 0.001$) establishing that F344-E animals obtain more cocaine reinforcers than F344-S animals but they respond at the same rate. **D.** HIV-1-S and HIV-1-E animals have parallel slopes, but have different intercepts $p \leq 0.01$ indicating that although equol treatment did not change the rate of responding, it did change consumption with HIV-1-S rats consuming more cocaine. **E.** Animals were given the opportunity to respond on a PR paradigm that introduced increasing concentrations of cocaine across testing days. All animals have a robust linear response $p \leq 0.006$; however, HIV-1 Tg animals do not have a non-zero slope, indicating that increasing concentrations of cocaine do not alter responding and F344 animals obtained significantly more infusions of cocaine at all concentrations ($p \leq 0.05$). Equol experience did not alter responding in either genotype. Data represents mean values ± 95% CI.

Experiment 3: Choice

All animals had a significant amount of experience with both cocaine and sucrose in an operant paradigm. The next experiment examined the ability of these animals to choose between reinforcers simultaneously. A 2 x 2 x 2 x 7 mixed ANOVA using number of lever presses was used to examine these results. There was a significant day x genotype interaction $F(6,84)=3.0$, $p \leq 0.05$ and a significant day x genotype x treatment interaction (6,84)=2.7, $p \leq 0.02$. Finally, there was a significant reinforcer type x treatment interaction $F(1,14)=11.1$, $p \leq 0.01$.

Regression analyses were used to further explore significant interactions. A history of equol treatment differentially modulated choice across days in both genotypes. F344-E animals had significantly different slopes $F(1,94)=4.5$, $p \leq 0.05$, indicating a differential response rate between reinforcers. F344-E animals had a functionally linear response to both reinforcers; animals preferred cocaine over sucrose, at a roughly 3:1 ratio. There was a negative slope for cocaine responding significantly different from zero $F(1,47)=6.3$, $p \leq 0.02$ ($r^2=0.89$), resulting in a decrease in preference by day 7 (figure 6.3C), but F344-E animals still preferred cocaine over sucrose at a 2:1 ratio. Meanwhile, F344-E animals did not change their response rate for sucrose over the course of choice

testing, with a slope that was not significantly different from zero ($p>0.1$; figure 6.3C). Replacement of cocaine with saline on day 8 resulted in an increase in responding for sucrose, roughly a 2:1 response for sucrose over drug. On day 9 when sucrose was replaced with water, there was not a significant preference for sucrose or drug.

Conversely, F344-S rats had significantly different intercepts $F(1,53)=12.5$, $p\leq0.001$ between reinforcer type but not a significant difference in slope, indicating that F344-S preferred sucrose over drug however they did not differ in their response rate (figure 6.3E). During extinction trials on days 8 and 9 there was not a significant shift in reinforcer preference.

HIV-1-E animals had significantly different slopes $F(1,76)=4.4$, $p\leq0.05$ with responding for sucrose having a significantly non-zero, positive slope $F(1,38)=4.5$, $p\leq0.05$ and the slope of drug response not being significantly different from zero, indicating that sucrose responding increased across testing days while drug response remained the same (figure 6.3D). HIV-1-S animals did not have slopes significantly different from zero for either reinforcer, however they had significantly different intercepts $F(1,55)=6.1$, $p\leq0.02$ indicating that HIV-1-S animals had a stable preference for sucrose over drug (figure 6.3F). Extinction trials on days 8 and 9 resulted in a decrease in responding for both sucrose and cocaine resulting in a lack of preference for both HIV-1-S and HIV-1-E groups.

Experiment 4: Dendritic Spine Analysis

Within 24 hours of the last cocaine self-administration session, animals were sacrificed and their tissue processed for identification of dendritic spines. Medium spiny neurons were identified and imaged (n=4-8 per animal). A Kruskal-Wallis test was

utilized to confirm that all cells analyzed were from similar populations. The null hypothesis failed to be rejected (p>0.4), indicating that all cells used for analysis were from the same population. Spine length, head diameter, and volume were analyzed using a 2x2 ANOVA with each parameter as the dependent variable.

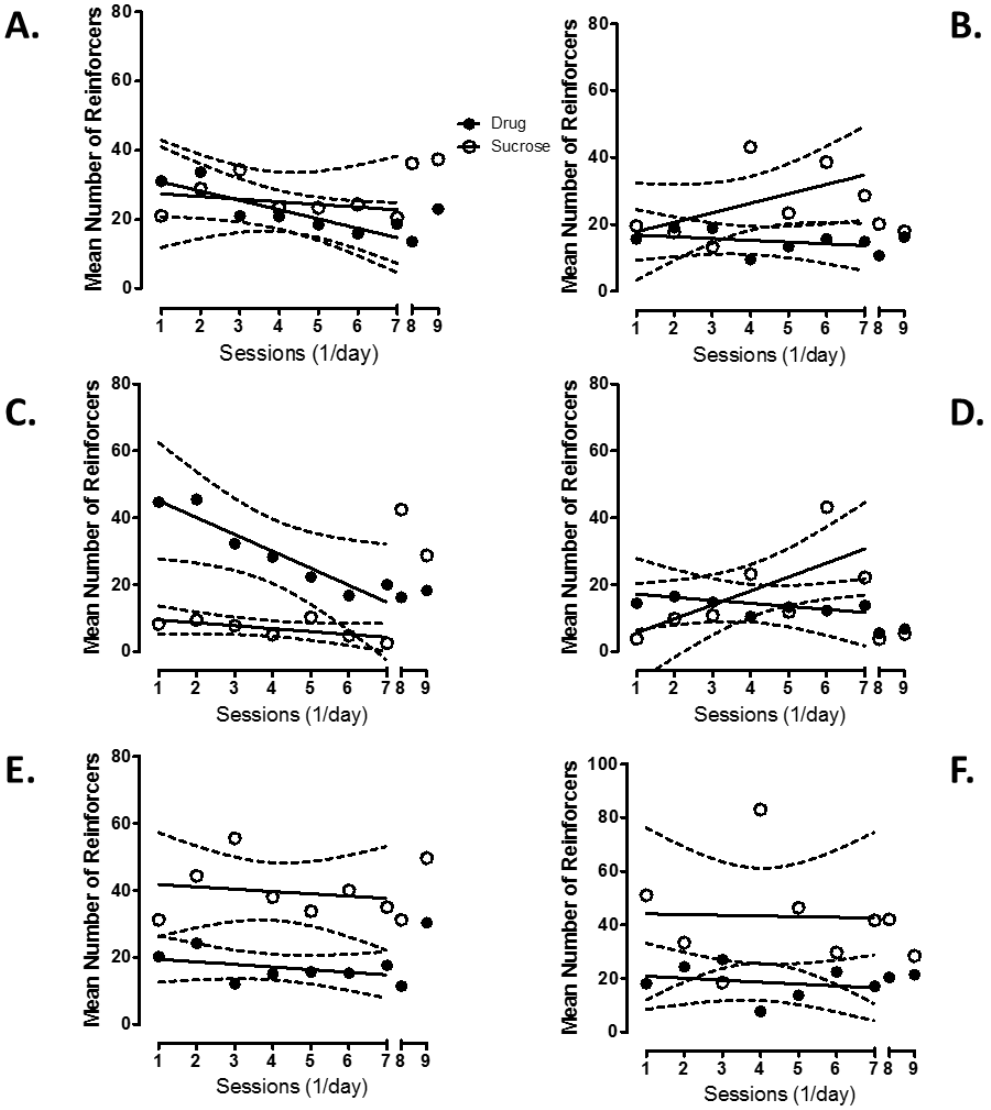

Figure 6.3 History of equol treatment modulates choice. **A.** When collapsed across treatment, F344 animals do not have a significant preference for drug or sucrose. Extinction trials (sessions 8[saline] and 9[water]) did not result in a preference shift. **B.** When collapsed across treatment HIV-1 Tg animals prefer sucrose over cocaine (p≤0.02). Extinction trials (sessions 8 [saline] and 9 [water]) did not result in a preference shift **C.**

F344-E animals have significantly different slopes for each reinforcer, $p \leq 0.05$. Cocaine was preferred over sucrose, however this is a significantly negative slope $p \leq 0.02$ resulting in a shift from a 3:1 preference to a 2:1 preference for cocaine, while sucrose responding was stable across testing days. Replacement of cocaine with saline resulted in an increase in responding for sucrose, resulting in roughly a 2:1 preference for sucrose over cocaine. **D.** HIV-1-E animals have significantly different slopes between reinforcer type $p \leq 0.05$, indicating a differential response for one reinforcer over the other. More specifically, HIV-1-E animals have a significantly positive slope for sucrose reinforcement while drug reinforcement remains stable, these slopes are significantly different from one another, $p \leq 0.05$, indicating HIV-1-E animals respond at different rates for sucrose and cocaine. Extinction trials (8 and 9) resulted in a decrease in sucrose responding resulting in a shift to non-preference between reinforcers. **E.** F344-S animals prefer sucrose over cocaine over the course of choice testing as evidenced by the significantly different intercept $p \leq 0.001$. Neither line was significantly different from zero, indicating a stable preferences for sucrose over cocaine. Replacement of cocaine with saline or sucrose with water did not alter choice preference. **F.** HIV-1-S animals do not have slopes significantly different from zero for either reinforcer, however their intercepts are significantly different $p \leq 0.02$ indicating a stable preference for sucrose over drug. Extinction trials (8 and 9) did not produce a preference shift. Data represents mean values ±95% CI.

Analyses revealed that F344 animals, regardless of treatment, had longer dendritic spines $F(1,56975)=142.1$, $p \leq 0.001$ (figure 6.4), with larger spine head diameter $F(1,48905)=33.9$, $p \leq 0.001$ (figure 6.5), than HIV-1 Tg rats. History of equol treatment produced a significant reduction in spine head diameter $F(1,38616)=25.9$, $p \leq 0.001$ (figure 6.5) and a corresponding loss of spine volume $F(1,13832)=12.2$, $p \leq 0.001$ (figure 6.6). There were significant genotype x treatment interactions for spine head diameter $F(1,38616)=35.3$, $p \leq 0.001$ (figure 6.5) and spine length $F(1,48905)=4.0$, $p \leq 0.05$. Equol treatment resulted in a decreased spine head diameter, and longer spines in F344 animals. However equol treatment resulted in increased spine head diameter, and marginally shorter spines in HIV-1 Tg animals.

There was a significant main effect of genotype on the complexity of branching in the MSNs of the NAc $F(1,51)=33.3$, $p\leq 0.001$, with F344 animals having a significantly more complex network than HIV-1 Tg animals (figure 6.7).

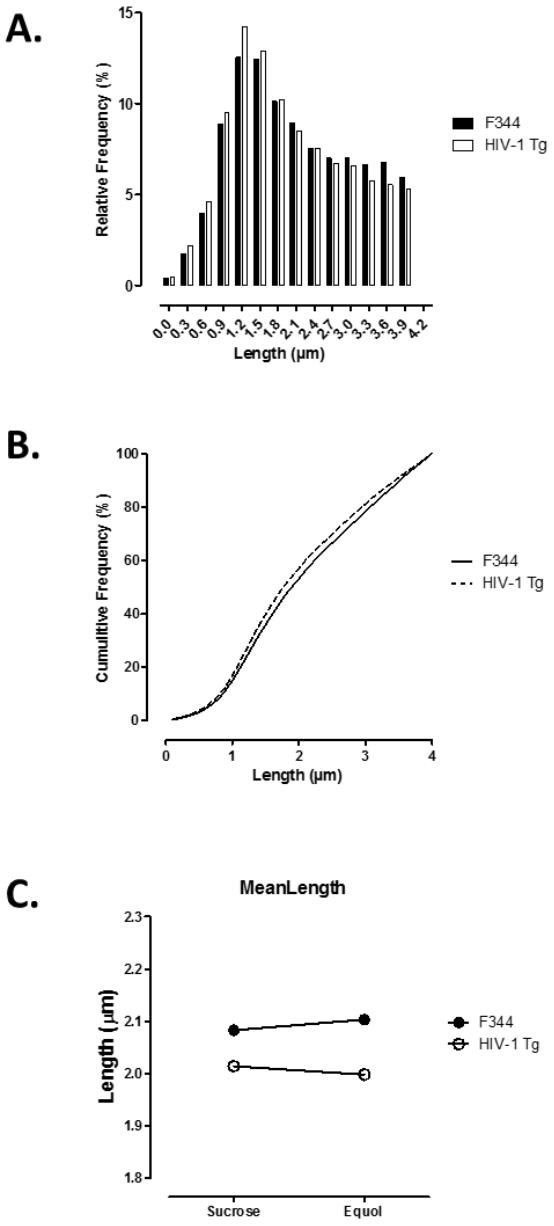

Figure 6.4 Spine length histograms illustrating **A.** relative and **B.** cumulative frequencies. **C.** Represents mean (±SEM) differences between genotypes. Regardless of treatment, there was an overall reduction in spine length in HIV-1 Tg animals reflecting a shift in

the population of spines observed. HIV-1 Tg animals have a shorter population of dendritic spines compared to F344 controls [$F(1,56975)=142.1$, $p\leq 0.001$].

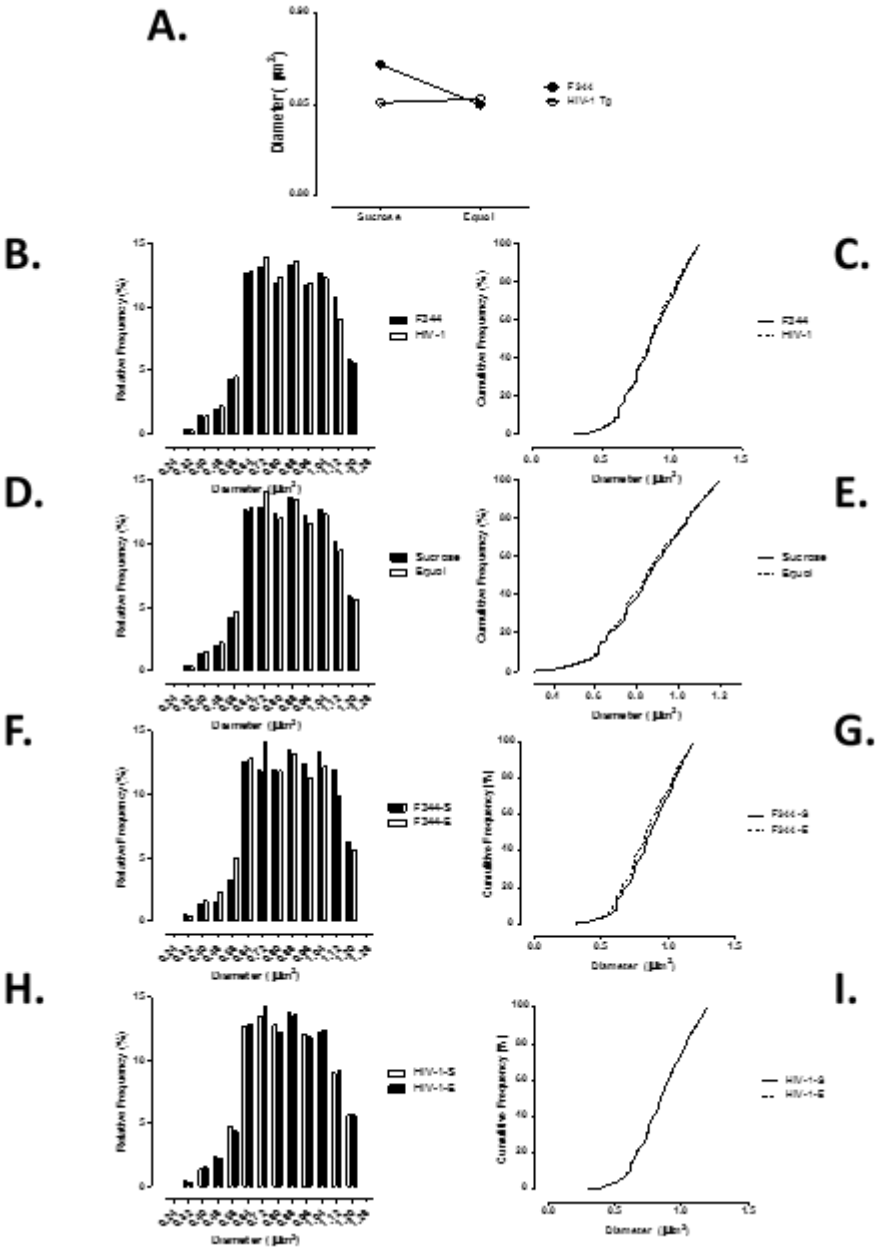

Figure 6.5 HIV-1 Tg animals have smaller spine head diameter relative to F344 animals and equol differentially modulates diameter. **A.** Mean (±SEM) plot illustrating the significant effect of genotype, treatment, and genotype x treatment interaction. **B.** Relative and **C.** cumulative frequencies of spine head diameter in HIV-1 Tg and F344 animals. HIV-1 Tg animals have a smaller spine head than F344 animals

F(1,48905)=33.9, $p \leq 0.001$. **D.** Relative and **E.** cumulative frequencies of spine head diameter as a function of treatment. Equol produces a shift in the population of spines; according to treatment. More specifically, equol treatment resulted in a significantly smaller spine head F(1,38616)=25.9, $p \leq 0.001$. **F**. Relative and **G.** cumulative frequency distributions of spine head diameter of F344-S and F344-E spines; Equol treatment results in a shift in the population of spines to smaller head diameter F(1,38616)=35.3 $p \leq 0.001$ in the F344 rat. **H.** Relative and **I.** cumulative frequency distributions of spine head diameter in HIV-1-S and HIV-1-E spines. Unlike F344 animals, there was not a significant shift in spine head diameter.

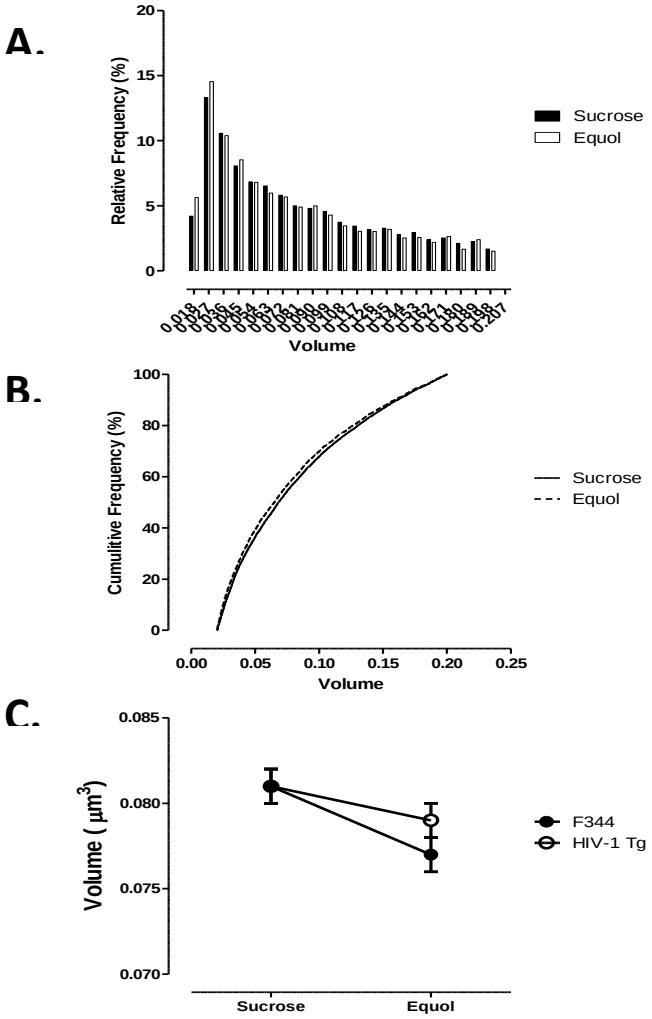

Figure 6.6 Equol modulates spine volume. **A.** Relative and **B.** cumulative frequency histograms illustrating the population shift of spine volume according with equol treated animals having lower volume spines, $F(1,13832)=12.2$, $p \leq 0.001$. **C.** Mean (±SEM) plot

illustrating the effect of equol treatment on spine volume. Regardless of genotype, spine volume was reduced in equol treated animals resulting in a shift in the population of spines observed.

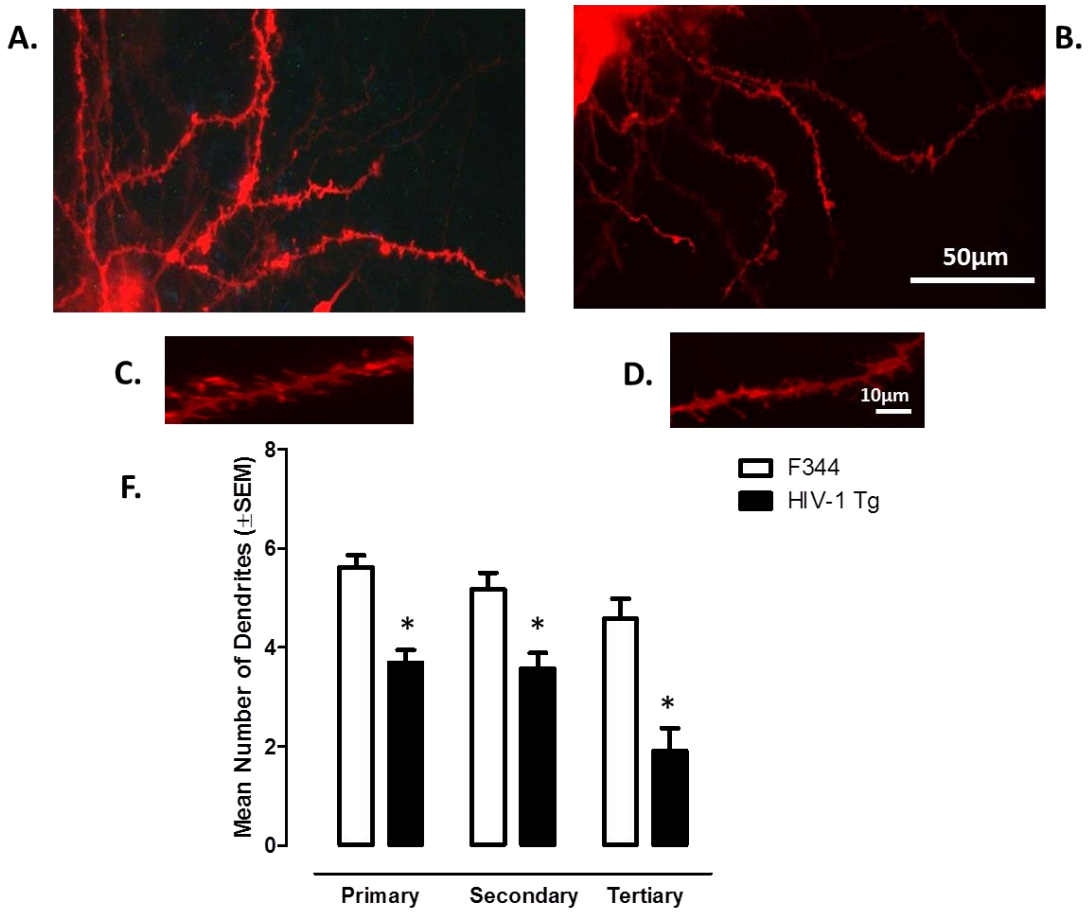

Figure 6.7 DiOlistic labeling of medium spiny neurons in the NAc. **A**. Representative F344 control image showing complex branching patterns resulting in an increased number of dendrites due to higher branch order (60x). **B.** HIV-1 Tg MSNs exhibit less complex branch order patterns, resulting in a stunted neuronal tree and potentially dysfunctional neural communication (60x). **C.** F344 animals displayed spines that were longer and with larger spine head diameter. **D.** HIV-1 Tg animals had shorter spines with less prominent spine heads. **F.** There was a significant main effect of genotype on dendritic branch order at the primary, secondary, and tertiary levels $F(1,51)=33.3$, $p \leq 0.001$.

Behavior predicting structural outcomes

An exploratory stepwise regression was used to determine the ability of performance on the behavioral tasks to predict individual spine parameters.

Spine head diameter. Performance on FR and PR sucrose tasks were determined to significantly predict spine head diameter in medium spiny neurons of the nucleus accumbens (data from each day were considered to be independent predictors, [$F(4,8)=15.6$, $p\leq 0.001$]). The adjusted r^2 indicated that 85.6% of the variance in spine head diameter was associated with number of sucrose reinforcers received during FR-5%, FR-30%, and PR-1%.

Length. Dendritic spine length was significantly predicted by performance on day 7 of choice, specifically by responding for the sucrose reinforcer $F(1,10)=8.8$, $p\leq 0.05$. The adjusted r^2 indicated that 46.7% of the variance in spine length was associated with performance on sucrose responding on day 7 of choice.

Volume. Dendritic spine volume was also significantly predicted by performance on both sucrose, cocaine self-administration, and choice behavior $F(4,7)=36.9$, $p\leq 0.001$. The adjusted r^2 indicated that 92.9% of the variance in spine volume was associated with the number of sucrose reinforcers received on PR30 and Day1 of choice; the number of cocaine reinforcers received in the 0.75mg/kg/inj dose in the cocaine dose response study and the first day of acquisition.

Discussion

Drug use, especially cocaine use (Cook et al., 2007; Korthuis et al., 2008), is a common comorbid condition with HIV-1+ serostatus (Ferris et al., 2008; Qian et al.,

2014; Purohit et al., 2011). HIV-1+ status alone is known to increase apathy and depression (Paul et al., 2005a; Paul et al., 2005b; Rabkin et al., 2000; Shapiro et al., 2013), decreasing the likelihood of medication adherence and disrupting quality of life (Shapiro et al., 2013; Tate et al., 2003). Current drug use plays a role in worsening neurocognitive deficits (Nath, 2010; Purohit et al., 2011), and there are no treatment options for these individuals. Presently, the HIV-1 Tg rat was used to evaluate the effectiveness of the gut metabolite equol on ameliorating alterations in response vigor for sucrose and cocaine. HIV-1 Tg rats display a decreased response rate for sucrose compared to F344 control rats, regardless of treatment status using a PR paradigm. Conversely, when an FR paradigm is used for testing there is a significant effect of equol treatment with equol treated animals responding more regardless of genotype. However, equol treatment differentially modulates cocaine acquisition and performance on the 14 day PR task in HIV-1 Tg rats versus F344 rats. Although treatment history does not alter responding for increasing concentrations of cocaine, it significantly alters choice behavior. Dendritic spine analysis revealed that HIV-1 Tg rats had significantly shorter spines with smaller head diameter, suggesting altered synaptic function. Additionally, history of equol treatment produced dendritic spines with smaller head diameter and less volume than in sucrose treated animals. Finally, performance on behavioral tasks was used to attempt to predict structural changes in dendritic spines. This revealed a complex set of predictors indicating that performance on both sucrose, cocaine, and choice tasks produce profound structural effects.

The HIV-1 Tg has an altered response for sucrose reinforcement on PR and FR schedules of reinforcement; specifically, response vigor is diminished. Equol treatment

did not enhance response vigor for sucrose during the PR task, however there was a significant effect of equol treatment resulting in an increase in sucrose rewards received in the FR task. Both HIV-1 Tg and F344 equol treated animals had increased responding for lower concentrations of sucrose but less for the highest concentration of sucrose, 30%. Although HIV-1-E rats respond more for sucrose than HIV-1-S rats, they still respond with significantly less vigor that either F344 group. The HIV-1 Tg rat also displays dopaminergic dysfunction (Moran et al., 2013; Moran et al., 2012), and HIV-1 proteins disrupt dopaminergic tone *in vivo* (Ferris et al., 2009a), and alter dopaminergic function *in vitro* (Ferris, Calipari, Yorgason, & Jones, 2013) and *in vivo* (Bertrand et al, 2015). The dopaminergic system plays an important role in motivated behavior (Baik, 2013; Robbins & Everitt, 1996) and the present results suggest that equol treatment may blunt responsiveness for highly appetitive reinforcement, like 30% sucrose, in control animals and modulate response vigor in HIV-1 Tg rats. Although equol treatment appears to modulate dopaminergic involvement in motivated behavior for sucrose, equol does not completely ameliorate deficits in motivational drive.

During the cocaine acquisition period equol treated animals initially respond more vigorously for cocaine, unlike sucrose treated animals which respond in a stable manner across five days; however, cocaine intake decreases over the 5 day acquisition period for equol treated animals. Animals had not received equol treatment for one week prior to the start of the acquisition period and did not receive treatment until after the first testing session. The initial up-regulation of responsiveness suggests that previous history with equol, a potent ERβ agonist, may increase response to novelty and/or increase initial sensitivity to the drug. However, as animals receive treatment over the 5 day acquisition

period there is a significant decrease in response suggesting that direct ERβ activation may play a significant role in acquisition. Estradiol is known to significantly increase acquisition of cocaine self-administration in OVX female rats (Hu et al., 2004; Hu & Becker, 2008), and estrogen modulates dopamine receptor expression in the VTA and midbrain (Zhou, Cunningham, & Thomas, 2002b). ERβ activation is involved in cocaine primed reinstatement (Larson & Carroll, 2007b), and here we demonstrate that ERβ may play an important role in the acquisition of cocaine self-administration.

Similar to the actions of cocaine, the neuroactive protein HIV-1 Tat inhibits DAT function (Midde et al., 2012; Midde et al., 2013) increasing the extracellular concentrations of dopamine. HIV-1 Tg rats self-administering cocaine have a slower dopamine turnover rate and V_{max} in the striatum (Bertrand, 2015). Together these results suggest that cocaine self-administration results in hyperdopaminergic tone, which is similar to what is found in rats treated with exogenous HIV-1 Tat and cocaine (Ferris et al., 2010), which may result in fewer responses for higher doses of cocaine. Presently, both HIV-1 Tg and F344 rats display a significant increase in cocaine intake on the 14 day PR schedule. However, HIV-1 Tg rats received more cocaine reinforcers than F344 rats overall, which is contrary to our previous report (Bertrand et al., 2015). In our previous study we showed that HIV-1 Tg rats had a modified response to cocaine, however, presently we illustrate that this may be due to an increased sensitivity to the drug itself. Indeed, a recent report describes the HIV-1 Tg rat as being more sensitive to the effects of cocaine at low doses (McIntosh et al., 2015). A lower dose of cocaine was used during the acquisition phase in order to more clearly differentiate the sensitivity of the HIV-1 Tg rat from the reinforcing efficacy of cocaine. Although HIV-1 Tg rats

respond more for a lower dose of cocaine, suggesting they may be more sensitive to lower cocaine doses, the reinforcing efficacy of the drug is also altered as demonstrated by the lack of a response to increasing doses of cocaine.

During the 14 day PR task, treatment with the potent ERβ agonist equol resulted in a differential modulation of behavior according to genotype. HIV-1-E rats take significantly fewer hits of cocaine compared to HIV-1-S animals over the two week time period; meanwhile F344-E rats take significantly more hits of cocaine compared to F344-S rats. Together, these results suggest that equol may have action in the dopaminergic system resulting in an alteration in dopaminergic tone. Once cocaine use is initiated, females are more likely than males to develop addiction and that estrogen plays a major role in this sex difference (Anker & Carroll, 2011). The role of estrogen in enhancing addiction indicates that estrogen has powerful effects on the dopamine system (Zhang, Yang, Yang, Jin, & Zhen, 2008). ERβ expression has been shown to alter DAT dynamics in the striatum (Al-Sweidi et al., 2012), and plays a role in the reinstatement process (Larson & Carroll, 2007b).

Activation of ERβ in HIV-1 Tg rats may result in a normalization of dopaminergic tone via DAT regulation, thus decreasing cocaine sensitivity. On the other hand, ERβ activation in F344 rats may result in dysfunctional dopamine regulation leading to an increased responsiveness to cocaine. Estradiol facilitates acquisition (Anker & Carroll, 2011; Jackson, Robinson, & Becker, 2006; Zhao & Becker, 2010), escalation (Larson, Anker, Gliddon, Fons, & Carroll, 2007), and reinstatement (Anker & Carroll, 2011). Estrogenic effects during reinstatement are ERβ dependent (Larson & Carroll,

2007a), and the present results suggest that ERβ activation may play an important role in regulating the escalation of cocaine intake in OVX F344 rats.

Drug addiction is commonly defined as a compulsive disorder, in which obtaining the drug occurs at the expense of obtaining natural reinforcers, such as food (Hyman, Malenka, & Nestler, 2006; Robinson & Berridge, 2003). Previously, we have shown that the HIV-1 genome disrupts the choice profile of the rat (see chapter 5). The equol effects in the choice paradigm occurred well after active treatment with equol had ended, thus, highlighting the long lasting behavioral effects of equol and the potential effectiveness of this gut metabolite to treat HIV-1+ cocaine users, but not seronegative cocaine users. History of S-equol treatment differentially modulates choice across days according to genotype. F344-E rats choose drug over sucrose suggesting an increase in compulsivity to obtain the drug; however, HIV-1-E rats choose sucrose over drug, similar to HIV-1-S and F344-S rats. Interestingly, F344-E rats almost triple their response for sucrose when cocaine is replaced with saline, and this increased responding does not change when sucrose is replaced with water.

Dendritic spines are dynamic structures that are thought to play major role in learning and memory (Sala & Segal, 2014; Segal, 2005). Alterations in dendritic spine morphology are correlated with neurotransmitter release (Murthy, Sejnowski, & Stevens, 1997), and spines with a large head diameter (i.e. mushroom spines) are more stable than thin or stubby spines (Segal, 2005). Although behavioral correlates spine morphology are not completely understood (Sala, Cambianica, & Rossi, 2008), there are specific spine phenotypes associated with neurochemical mechanisms. We demonstrate that HIV-1 Tg rats have shorter spines, with smaller head diameter, and less volume compared to F344

rats, suggesting that HIV-1 Tg rats have less mature spines and/or transient, dysfunctional synapses present consistent with our previous findings (Roscoe, Jr. et al., 2014). There was no significant treatment effect in HIV-1 Tg rats; however, history of equol resulted in dendritic spines with a smaller head diameter and overall volume in F344 animals indicating that equol produced a long lasting structural effect. The equol induced alterations in spine head diameter and volume suggest a less stable spine phenotype than non-equol treated controls.

History of equol treatment did not significantly alter dendritic spine parameters in the HIV-1 Tg rat, potentially due to the cessation of equol treatment 4-5 weeks prior to sacrifice. Although equol treatment ended, it is important to note that cocaine self-administration continued. HIV-1 proteins and cocaine result in enhanced synaptodendritic injury (see chapter 4) and neuronal cell death *in vitro* (Kendall et al., 2005; Turchan et al., 2001). The present results suggest that cocaine and HIV-1 proteins interact and may produce morphological changes consistent with a less stable phenotype (Segal, 2005).

Previously we reported that HIV-1 Tg rats have significantly shorter dendritic spines with a decreased volume, indicating a shorter stubbier spine phenotype (Roscoe, Jr. et al., 2014). Presently we demonstrate a similar dendritic spine phenotype, with HIV-1 Tg rats having shorter spine length and a reduced spine head however there was not a significant change in overall spine volume between genotypes. Estrogen is known to effect spine head diameter and spine volume (Velazquez-Zamora et al., 2012; Brocca, Pietranera, Beauquis, & De Nicola, 2013), which may explain differential findings of dendritic spine parameters in our present study and our previous study. The present experiment used OVX females self-administering cocaine under operant conditions,

while our previous report utilized intact females allowing estrogen mediated spinogenic effects to occur. Additionally, the rats in the present experiment also performed tasks to receive palatable reinforcement which is known to alter dendritic spine morphology (Guegan et al., 2013; Pitchers et al., 2010; Pitchers et al., 2013). Moreover, cocaine self-administration is also known to effect dendritic spines (Pitchers et al., 2013; Dobi, Seabold, Christensen, Bock, & Alvarez, 2011; Robinson & Kolb, 2004; Robinson & Kolb, 1999). Cocaine self-administration in the HIV-1 Tg rat results in significant alterations in dendritic morphology and that the interactions between neurotoxic HIV-1 proteins and cocaine may overcome the effects of equol treatment. The morphological results presented here suggest that HIV-1+ drug abusers may need more intensive treatment to alter structural changes induced by cocaine self-administration in the presence of HIV-1.

Morphological data were employed to predict performance on behavioral tasks, revealing a complex relationship between behavior and dendritic spines. Performance on a specific behavioral task did not uniformly predict morphological alterations, however, performance exclusively on sucrose associated tasks predicted spine head diameter and length. Conversely, spine volume was predicted by performance on both sucrose and cocaine tasks. Together these indicate that experience with natural rewards in an operant paradigm produces a long term morphological change that may contribute to cocaine taking behavior. Moreover, the morphological changes in the NAc predicting the response vigor for sucrose are consistent with the literature (Salamone & Correa, 2012).The complex associations between behavior and dendritic morphology are consistent with the current literature (Sala & Segal, 2014), and may require more direct

quantifications of behavior to better understand the predictive nature of morphological changes in operant tasks with both natural and drug reinforcement.

Our results illustrate the potential of the gut metabolite equol to treat motivational alterations in HIV-1. These results may translate into humans, suggesting that the gut microbiome plays an important role in treating HIV-1 associated neurocognitive disorders. Although equol appears to be a potential therapeutic candidate for HIV-1+ drug users, this does not appear to be a treatment appropriate for seronegative cocaine users. Equol treatment increased sensitivity and responsivity to lower doses of cocaine in F344 animals, suggesting equol alters dopamine signaling in normal rats as well as HIV-1 Tg rats. Interestingly, equol treatment also blunted the response for the highest dose of sucrose, 30%, compared to sucrose treated rats across genotypes. This suggests that equol, or gut metabolites in general, may alter the reinforcing properties of foods high in sugar as well as responsiveness to cocaine. The gut metabolite equol has long lasting effects on neuronal structure and may play an important role in treating HIV-1+ cocaine abuse.

CHAPTER 7

GENERAL DISCUSSION

High HIV-1 viral load are found in brain regions that regulate motivation, and alterations in motivated behavior are predictive of neurocognitive status in HIV-1 (Wiley et al., 1998; Berger & Nath, 1997; Chang et al., 2008). Neuronal damage and dysfunction in dopamine-rich areas are also predictive of neurocognitive status pre-mortem (Chang et al., 2008; Wang et al., 2004; Silvers et al., 2006). Phytoestrogens both prevent and restore synaptodendritic damage induced by HIV-1 Tat *in vitro* (see chapters 3 and 4). The combination of a sub-toxic dose of HIV-1 Tat and cocaine resulted in a significant loss of synaptodendritic integrity, and both enantiomers of equol prevented synaptodendritic injury induced by Tat+cocaine (see chapter 4). Similar to an HIV-1+ individual, the HIV-1 Tg rat has dopaminergic alterations; The HIV-1 Tg rat displays deficits in goal-directed motivated behavior (see chapters 5 and 6) and alterations in both dopamine transporter (DAT) function (see chapter 5) and dendritic spine morphology (see chapter 6)(Roscoe, Jr. et al., 2014). Treatment with the gut metabolite equol altered motivated goal-directed behavior in HIV-1 Tg rats for sucrose and cocaine (see chapter 6).

Motivation is fundamental to behavior, however it is not formally considered within the domain of cognition (Keeler & Robbins, 2011). Undeniably, the neural processes that mediate motivation are critical for multiple aspects of cognitive function. The construct of motivation is widely used in behavioral neuroscience research, although

it has been given very little attention with respect to HIV-1. Motivation is defined as a state that allows an animal to regulate its internal and external environments through the organization of its behavior (Koob, 2009; Salamone & Correa, 2012). Goal-directed behavior, which encompasses Pavlovian and operant conditioning processes, is one example (Rescorla, 1987). The function of goal-directed behavior is to bring environmental contingencies under control, and it requires, at minimum, motivation, associative learning processes, and memory, e.g., stimulus-outcome associations (incentive wanting;Robinson & Berridge, 2003), stimulus-response associations (habit; Everitt & Robbins, 2013) and response-outcome associations (Adams & Dickinson, 1981; Rescorla, 1987). Once learned, goal-directed behavior is maintained by motivational and cognitive processes, such as the animals' homeostatic state, an expectation about the reinforcer, the current value of the reinforcer, environmental cues, and other learning phenomena, such as generalization and discrimination (Spear & Riccio, 1994; Koob, 2009).

Apathy operationalized as a reduction in self-initiated and goal-directed behavior, or a lack of motivation (Marin, 1991). HIV-1+ individuals consistently report higher incidence of apathy than seronegative controls (Kamat et al., 2012; Castellon et al., 1998a; Paul et al., 2005a). Elevated apathy scores are predictive of decreased medication adherence (Panos et al., 2014), more cognitive complaints, decreased neurocognitive performance (Shapiro et al., 2013; Kamat et al., 2012), and diminished everyday functioning (Kamat et al., 2012; Kamat et al., 2013). In HIV-1 positive individuals, elevated levels of apathy are related to alterations in learning efficiency and cognitive

flexibility (Paul et al., 2005a), in addition to decreased working memory performance (Castellon et al., 1998a) and executive dysfunction (Castellon et al., 2000).

The construct of apathy is not frequently discussed in animal models; however the concept of motivation, or how willing an animal is to work for reinforcement, is frequently tested in animals using operant techniques (Caine & Koob, 1994b; Harrod et al., 2012a; Lacy et al., 2014; Lacy et al., 2012a). However, this only evaluates one facet of motivation – specifically, vigor, or effortful work in order to obtain a reinforcer. Consummatory behavior, or direct interaction with the reinforcer, also encompasses motivated behavior; and this aspect of motivation is not disrupted by dopamine. Fixed ratio (FR) schedules of reinforcement, where animals perform a fixed set of responses for reinforcement, generally only assesses how much of a particular reinforcer is consumed in a particular session. In the case of drug reinforcement these results may be skewed if there are varying doses because a higher dose would result in fewer responses, however, the higher dose is presumably more reinforcing. In order to more easily interpret operant results, frequently a progressive ratio (PR) schedule of reinforcement is used. The amount responses required to obtain a reinforcer are increased following each successful ratio completion, eventually resulting in a break point where the animal stops responding. Generally, the higher the break point, the more valuable a particular reinforcer.

Apathy is defined as a substantial lack of motivation, operationalized by diminished goal-directed behavior, diminished goal-directed cognition, diminished concomitants of goal directed behavior (Starkstein & Leentjens, 2008), a reduction in emotional engagement, reduced interest in normal purposeful behavior, and problems in beginning of purposeful movement (Moretti, Cavressi, & Tomietto, 2014). Apathy is an

important facet of HIV-1 associated neurocognitive disorders as it greatly impacts the quality of life in HIV-1+ individuals (Kamat et al., 2012; Kamat et al., 2013; Shapiro et al., 2013), and is a major predictor in medication adherence (Panos et al., 2014). A reduction in seeking or approach behavior, e.g. operant performance for sucrose, has been used to measure apathy in animal models of neurodegenerative disorders like Parkinson's disease (Favier et al., 2014; Martinowich et al., 2012). However, this is only one aspect of motivation and thus only measures a small portion of apathy symptomology. Further studies examining primary reinforcer intake (e.g. food) independent of operant tasks, social engagement, locomotor activity, and response to novelty may increase the understanding of apathy in an animal model.

Dysfunction of, and damage to, the frontal-subcortical system is believed to play a role in manifestation of apathy (Masterman & Cummings, 1997; Tekin & Cummings, 2002). The basal ganglia play an important role in the initiation of motivated behavior, voluntary movement, and learning stimulus-response associations. Damage to the striatum and the basal ganglia impair the ability of animals (McDonald & White, 1994) and humans (O'Doherty et al., 2004; Knowlton, Mangels, & Squire, 1996) to learn stimulus-response associations. Reports of apathy in HIV-1 positive individuals are associated with decreased performance on tasks known to involve the frontal-subcortical circuitry (Cole et al., 2007), and lower overall volume of the nucleus accumbens (Paul et al., 2005b). Areas in the mesocorticolimbic system are dopamine rich and are known to be more susceptible to HIV-1 infection (Berger & Nath, 1997; Nath et al., 2000; Wiley et al., 1998). HIV-1+ patients have decreased DAT labeling in the striatum (Chang et al.,

2008), fewer TH stained neurons at autopsy (Silvers et al., 2006), and parkinsonian-like symptoms (Tse et al., 2004).

The DAT in the striatum of the HIV-1 Tg rat are dysfunctional, which may result in an altered dopaminergic tone. In drug naïve animals, HIV-1 Tg rats have a lower binding coefficient compared to F344 control animals, indicating fewer DAT on the membrane. However, these DAT act more efficiently, as the V_{max} is higher in HIV-1 Tg rats than F344 control rats, resulting in a higher dopamine turnover rate at baseline. In HIV-1 Tg animals that have a history of cocaine self-administration, these results are reversed – the binding coefficient increases, but V_{max} decreases resulting in a significantly lower dopamine turnover rate, potentially translating to a hyperdopaminergic tone within the synapse compared to F344 rats. Indeed, previous work has demonstrated that HIV-1 Tat and cocaine (i.p. injection) result in hyperdopaminergic tone (Ferris et al., 2010) and impaired DAT function (Ferris et al., 2009b).

Dysfunctional modulation of dopamine release and uptake may result in an up-regulation of free radical production. HIV-1 Tat decreases surface expression of the DAT and diminishes the function of vesicular monoamine transporter (VMAT-2) (Midde et al., 2012) and produces a reversible conformational change of the DAT in striatal synaptosomes (Midde et al., 2013). The interactions of HIV-1 Tat with the DAT, in addition to the acute inhibition of the DAT by cocaine, may result in a prolonged period of synaptic DA exposure. Additionally, inhibition of VMAT-2 suggests that DA that has been successfully removed from the synapse is not packaged into vesicles. Overall, inhibition of the DAT and VMAT-2 allow for an increased likelihood of oxidized dopamine.

The prolonged exposure of DA in the intra- and extra-cellular space may result in an increase in oxidative damage. Indeed, HIV-1 proteins lead to an increase in oxidative stress in primary hippocampal cell cultures (Aksenov et al., 2006) and there is an increase in protein oxidation in animals acutely treated with HIV-1 Tat (Aksenov et al., 2003; Aksenov et al., 2001). Thus, it is plausible that in the current studies that the HIV-1 Tg rats self-administering cocaine, and primary neuronal cultures treated with Tat + cocaine, have elevated MAO-A levels resulting in an increase in protein oxidation and cellular damage above and beyond Tat induced changes on the DAT. The final result may be that HIV-1 rats have an altered choice profile and altered reinforcing efficacy of cocaine. Morphologically, the increase in oxidative damage may result in depolymerization of F-actin, causing the reduction of F-actin rich structures observed in the *in vitro* studies, and alterations in dendritic spine morphology *in vivo*.

Dendritic spine morphology is correlated with spine function (Segal, 2005), with enlargement of spine head diameter associated with long-term potentiation (Sala & Segal, 2014; Segal, 2005). In the NAc and striatum medium spiny neurons play a major role in mediating reward and reinforcement (Yagishita et al., 2014). MSNs receive input from excitatory neurons and modulatory (e.g. dopamine) neurons. Not only is dopamine required for MSN spine head enlargement, but the precise timing of dopamine release is also a major player in the degree of spine head enlargement (Yagishita et al., 2014). Timing of reward presentation has been long known to be important in instrumental conditioning (Benjamin & Perloff, 1983), and this appears to be a molecular correlate of reinforcement driven behavior. HIV-1 and cocaine significantly alter DAT functionality which results in altered release and re-uptake of dopamine, potentially disrupting ideal

timing of dopamine interaction with the dendritic spine head. Indeed, we see that the HIV-1 Tg rat (control) has significantly smaller spine head diameter, which may indicate that HIV-1 Tg rats have less robust formations of LTP in the NAc resulting in altered processing of reinforcement.

The persistent change seen in addiction requires a strong association and an increase in synaptic strength, accompanied by a structural change in the axon or dendrite (Chklovskii, Mel, & Svoboda, 2004). LTP and LTD play an important role in experience-dependent learning and memory (Papper, Kempter, & Leibold, 2011), and are mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) receptor, as well as metabotropic glutamate receptors (mGluR). HIV-1 Tat is known to interact with NMDA receptors (Aksenov et al., 2012; Li et al., 2008), potentially disrupting the formation of LTP in the NAc resulting in a smaller spine head diameter.

In the *in vitro* studies presented in this dissertation, there was a loss of F-actin puncta density following either a 24 hour incubation with HIV-1 Tat (1-86B, 1-72B, 1-101B; 50 nM) or HIV-1 Tat (1-86, 10 nM) + cocaine (1.6 µM). This is consistent with *in vivo* studies which report a loss of dendritic spine density in humans (Atluri et al., 2013) and animal models (Fitting et al., 2010b). F-actin is a major cytoskeletal protein that is found in both pre- and post-synaptic structures (Johnson & Ouimet, 2006). F-actin is found in both the spine head and shaft (Sekino et al., 2007; Dent et al., 2011), filopodia (long, headless, "pre-spines") (Jacinto & Wolpert, 2001; Hotulainen et al., 2009; Kaech et al., 2001; Sekino et al., 2007), and in patches along the dendrite (Halpain et al., 1998). Patch morphology appears both at the initiation of spinogenesis (Johnson & Ouimet,

2006), and as non-spiny synapses (Craig et al., 1994; van Spronsen & Hoogenraad, 2010). Thus, *in vitro* changes in F-actin puncta density may reflect an inability of the neuronal network to maintain plastic potential.

In chapter 4, equol pre-treatment prevented loss of F-actin rich puncta induced by both toxic HIV-1 Tat doses (50 nM) and sub-toxic HIV-1 Tat doses (10 nM) + cocaine (1.6 µM) via an estrogen receptor dependent mechanism. A subsequent experiment determined that the neuroprotective properties of both equol enantiomers were mediated through the ERβ subtype. ERβ expression has been found in dopaminergic rich regions like the substantia nigra, basal ganglia (Shughrue et al., 1997b; Laflamme, Nappi, Drolet, Labrie, & Rivest, 1998), NAc (Shughrue et al., 1997b), and rostral portions of the VTA where it is co-localized with TH (Creutz & Kritzer, 2002b). ERβ immunopositve cells in the VTA and substantia nigra project to the striatum and NAc (Creutz & Kritzer, 2004). Estradiol upregulates D2 receptor and DAT density in the NAc via an ERβ dependent mechanism (Le Saux, Morissette, & Di, 2006; Le Saux & Di, 2006). The behavioral experiments described in chapter 6 demonstrated that equol resulted in complex behavioral and morphological changes in both HIV-1 Tg and control F344 rats, which may be mediated by the activation of ERβ in the VTA.

Equol may be potentiating communication between the VTA and the NAc following cocaine self-administration, contributing to the increase in response that is observed in F344 control animals for cocaine. Under normal conditions, like those present in the F344 control rats, activation of ERα or ERβ results in an increased response of the VTA to amphetamine (Sarvari et al., 2014). In HIV-1 Tg rats, this may

effectively ‘normalize’ the dopaminergic tone or may induce transcription of functional DATs.

Drug addiction is frequently classified as a compulsive need for the drug, where drug associated activities (e.g. acquiring and administering the drug) take center stage and nondrug activities (e.g. work, family obligations) are secondary (Hyman et al., 2006; Robinson & Berridge, 2003). Although self-administration models demonstrate increased drug intake (escalation; Ahmed & Koob, 1999; Morgan et al., 2006), animals are only given the option of responding on levers within the operant chamber. Seminal research in rhesus macaques demonstrated that when given the opportunity to respond for cocaine or food, cocaine was the preferred choice (Negus, 2003; Nader & Woolverton, 1991; Woolverton & Balster, 1979; Aigner & Balster, 1978). Conversely, more recent studies in rodent models have illustrated that rats and mice prefer sucrose or saccharin reinforcement over cocaine reinforcement (Cantin et al., 2010; Lenoir, Serre, Cantin, & Ahmed, 2007; Lenoir, Augier, Vouillac, & Ahmed, 2013).

The results in the choice paradigm discussed in chapters 5 and 6 present two different responses to choice between sucrose and cocaine. In chapter 5, we find contrary results in F344 control rats compared to the current literature (Cantin et al., 2010; Lenoir et al., 2007; Lenoir et al., 2013). Although F344 control rats initially prefer sucrose, over a period of days they shift to preference for cocaine, nearly 2:1 over sucrose. Similar to the prior literature (Cantin et al., 2010; Lenoir et al., 2007), our rats had extensive histories with both reinforcers prior to exposure to the choice procedure. However, the dose of cocaine used in our studies was determined by deriving the EC50 of cocaine and

sucrose from dose-response tasks prior to the choice task. Using the optimal concentration for both reinforcers may have produced an alteration in choice profile.

On the other hand, F344 control animals in chapter 6 display a relatively stable (e.g. slopes do not differ from zero) 2:1 preference for sucrose over cocaine. Both sets of animals had extensive experience with both sucrose and cocaine reinforcement and were explicitly trained to anticipate reinforcement from either active lever (e.g. no side bias). However, unlike in chapter 5, it was not possible to determine the drug EC50 for either genotype, so the training dose was used. The sucrose training dose (5% w/v) was also used for sucrose, in order to compare the choice effectiveness of training doses. The training dose in chapter 6 (0.2 mg/kg/inj) was lower than both the training dose and EC50 dose (0.33 mg/kg/inj) used in the choice paradigm in chapter 5. The differential results presented between these two studies may exhibit that although cocaine is preferred by OVX F344 rats, it is a dose-dependent preference. Additionally, the use of optimal concentrations for both drug and non-drug reinforcers may drive choice preference results.

HIV-1 Tg control rats also display differential results between the two studies presented in chapters 5 and 6. However, this is more prominent in chapter 5, where HIV-1 Tg animals initially prefer sucrose over cocaine, however over the choice period they decrease their sucrose responding and no longer have a preference between either reinforcer at the end of testing. On the other hand, HIV-1 Tg sucrose treated animals in chapter 6 prefer sucrose over cocaine, albeit not as pronounced as F344 sucrose-treated rats. The difference in response between studies may be contributed to the different doses of cocaine used throughout the study. In addition to the altered response of HIV-1 Tg

sucrose-treated rats in the acquisition, 14-day PR, and dose-response tasks in chapter 6 compared to chapter 5 suggest that the HIV-1 Tg rat may be more sensitive to lower concentrations of cocaine, similar to what was found in a recent study (McIntosh et al., 2015). Together the results of equol on choice indicate that equol has a long lasting behavioral effect, and may have detrimental properties in the non-HIV-1+ population. The effects of equol in the HIV-1 Tg rat require more evidence to reach a concrete conclusion.

In order to reach more firm conclusions in regards to the effects of equol on HIV-1, and evaluating the construct of motivation in HIV-1, in addition to the present studies ongoing in the lab (e.g. locomotor activity, wheel running), more experiments must be done. In order to more fully examine the behavioral effects of equol, a more consistent treatment schedule during cocaine self-administration should be performed. Due to supply limitations, following surgery equol was only given every other day until the end of the 14-day PR period. Due to the interactive effects of HIV-1 proteins and cocaine (see chapter 4; Kendall et al., 2005; Turchan et al., 2001) and low dose of equol used the morphological changes may not have been captured. The evidence of long-term morphological changes in the F344 rat provides evidence that long lasting structural changes occur in equol treated animals.

Second, it would be useful to evaluate the effects of equol treatment on sucrose preference using a five bottle test, similar to what was used in chapter 5. Preference testing may allow us a better understanding of the effects of equol on consummatory behavior, giving a more complete picture of the alterations of equol treatment on

motivation for sucrose solutions. Finally, using a higher dose of equol may alter response vigor of the HIV-1 Tg rat for sucrose, ameliorating the deficits noted in chapters 5 and 6.

It was not until recently that the gut microbiome was examined in any kind of depth. Nearly 15 years ago, the word gut microbiome was coined (Lederberg, 2001). Since then technology has increased exponentially allowing scientists to delve more deeply into the bacteria that inhabit the humans. Recently, the bidirectional communication between the resident gut microbiota and their effects on behavior were described as the 'gut-brain axis' (Cryan & Dinan, 2012; Foster & McVey Neufeld, 2013). Modulation of the gut microbiota with probiotic treatment have been successful treatments for anxiety-like and depressive-like behaviors in rats and mice (Neufeld et al., 2011a; Neufeld et al., 2011b). Probiotic treatment has also been suggested as a treatment for inflammatory bowel syndrome (IBS), a gastrointestinal disorder linked to increased stress levels (Clarke et al., 2012). However, the effects of the gut microbiota on diseases like HIV-1 have not been explored.

HIV-1 is currently a pandemic, however, there are higher incidences of HAND in individuals in North America and Europe as opposed to Sub-Saharan Africa and Asian populations (Satishchandra et al., 2000). Although these regional differences are generally believed to be due to viral strain differences, some variability may be due to differences in gut microbiota and diet. The gut microbiota may play an important role in mediating disease progression as well as the neurocognitive correlates of HIV-1. The ability of the gut metabolite equol to alter neuronal morphology and behavior in the studies in this dissertation suggests that the gut and diet may play an important role in modulating CNS health in HIV-1 and potentially other disease states.

Cocaine use during HIV-1 infection results in an increased incidence and severity of HAND (Baum et al., 2009; Chang, Connaghan, Wei, & Li, 2014; Nath et al., 2001; Nath, 2010). HIV-1 Tat and cocaine produce interactive damage on neurons *in vitro* (chapter 4), and attenuates the escalation of drug intake (chapter 6) suggests that treatment with equol may promote sobriety and protect from further neurological insult. The neuroprotective properties of equol, and the restorative properties of the parent compound daidzein (chapter 3), indicate that equol may be a potential therapeutic for HAND. Equol treatment in the HIV-1 Tg rat did not provide concrete answers on the effects of equol treatment on motivation. However, equol does not appear to be an optimal treatment for the HIV-1 negative cocaine using population, as equol appears to increase response to lower doses of cocaine, and may potentiate aberrant choice behavior. Importantly, because equol is a naturally occurring compound translation in to clinical testing would be faster than most therapeutic developments. Treatment with equol at HIV-1 diagnosis may reduce drug intake and prevent neuronal injury, however treatment may need to be constant or higher for positive effects. The effectiveness of equol may open the door to the exploration of the interactions of HIV-1 and the resident microbiota.

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